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Application de l'immunologie moderne au développement de vaccins contre les maladies vétérinaires tropicales

Mémoire présenté en vue de l'obtention de
l'habilitation à diriger des recherches

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LIEU ET DATE DE NAISSANCE : Liège, le 20 septembre 1964

NATIONALITE: belge

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Etudes, titres, diplômes

- 1994: Doctorat en Sciences, Université Libre de Bruxelles, Laboratoire de Biotechnologie des Cellules Animales, Prof. John Wérenne, Grande Distinction (16/20). Intitulé de la thèse: "Evaluation du rôle joué par les cytokines et la myeloperoxidase dans les infections par la rickettsie *Cowdria ruminantium*: implications dans les mécanismes de résistance et dans la pathologie".

- 1988: Ingénieur en Agronomie (Orientation Agronomie Tropicale), Université Libre de Bruxelles, Grande Distinction

Expérience professionnelle

- octobre 2003: Affecté au CIRAD-EMVT à Montpellier (en attente d'expatriation), programme santé animale. Immunologie et vaccinologie appliques à la PPCB (péripneumonie contagieuse bovine, infections à *Mycoplasma mycoides subsp.mycoides* SC)

- 1997: Affecté au CIRAD-EMVT, programme santé animale, mission Guadeloupe. Immunologie et vaccinologie appliques à la cowdriose dans le modèle caprin.

- 1994: Détaché par le CIRAD-EMVT à l' 'International Livestock Research Institute (ILRI)', Nairobi, Kenya. Immunologie cellulaire appliquée à la cowdriose (infections à *Ehrlichia ruminantium* (Ex. *Cowdria*) chez le bovin.

- chercheur permanent au département d'élevage et de médecine vétérinaire tropicale du Centre international en recherche agronomique pour le développement (CIRAD-EMVT) depuis 1994.

Publications (revues avec bureau de lecture)

12) I. Esteves, K. Walravens, N. Vachiery, D. Martinez, J.J. Letesson, and **P. Totté. 2004**. Protective killed *Ehrlichia ruminantium* vaccine elicits IFN-γ responses by CD4+ and CD8+ T lymphocytes in goats. **Veterinary immunology and immunopathology** 98; 49-57.

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10) K.T. Ballingall, D.M. Mwangi, N. MacHugh, E. Taracha, **P. Totté**, and D.J. McKeever. **2000**. A highly sensitive, non-radioactive assay for T-cell activation in cattle: applications in screening for antigens recognised by CD4+ and CD8+ T-cell lines and clones. **Journal of Immunological Methods** 239; 85-93.

9) N. Vachiéry, **P. Totté,** V. Balcer, D. Martinez, and A. Bensaid. **1999**. Effect of isolation techniques, *in vitro* culture and IFNγ treatment on the constitutive expression of MHC class I and class II molecules on goat neutrophils. **Veterinary Immunology and Immunopathology** 70 (1-2), 19 – 32.

8) **P. Totté,** A. Bensaid, S. Mahan, D. Martinez, and D. McKeever. **1999**. Immune responses to *Cowdria ruminantium* infections. **Parasitology Today** 15, no. 7(169), 286 –290.

7) **P. Totté,** J. Nyanjui, A. Bensaid, and D. McKeever. **1999**. Bovine CD4+ T-cell lines reactive with soluble and membrane antigens of *Cowdria ruminantium*. **Veterinary Immunology and Immunopathology** 70 (3–4), 269 – 276.

6) N. Vachiéry, I. Trap, **P. Totté,** D. Martinez, and A. Bensaid. **1998**. Inhibition of MHC class I and class II cell surface expression on bovine endothelial cells upon infection with *Cowdria ruminantium*. **Veterinary Immunology and Immunopathology** 61, 37 – 48.

5) **P. Totté**, D. McKeever, D. Martinez, and A. Bensaid. **1997**. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. **Infection and Immunity** 65, 236 - 241.

4) C. Tournay, P.J. Courtoy, L. Marodi, **P. Totté**, J. Wérenne, A. Jacquet, L. Garcia-quintana, A. Bollen, and N. Moguilevski. **1996**. Uptake of recombinant myeloperoxidase, free or fused to Fcγ , by macrophages enhances killing activity toward micro-organisms. **DNA and Cell biology** 15 (8), 617-624.

3) **P. Totté**, N. Vachiéry, H. Heremans, A. Billiau, and J. Wérenne. **1994**. Protection against *Cowdria ruminantium* infection in mice with gamma interferon produced in animal cells. *In* T. Kobayashi, Y. Kitagawa, and K. Okumura (eds.), **Animal cell technology: basic and applied aspects**, vol. 6: 595-599.

2) **P. Totté**, N. Vachiéry, D. Martinez, I. Trap, K.T. Ballingall, N.D. MacHugh, A. Bensaid, and J. Wérenne**. 1996**. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce MHC class II following infection of endothelial cells. **Veterinary Immunology and Immunopathology** 53, 61 - 71.

1) **P. Totté,** F. Jongejan, A.L. de Gee, and J. Wérenne. **1994**. Production of alpha interferon in *Cowdria ruminantium*-infected cattle and its effect on infected endothelial cell cultures. **Infection and Immunity** 62: 2600 - 2604.

Stages et collaborations

- début 1994 à fin 1996: post-doc dans l'équipe du Prof. D. McKeever à l'ILRI (International livestock research institute, Nairobi, Kenya). Ce séjour m'a permis d'acquérir une expérience en immunologie vétérinaire. Mes travaux sur l'immunologie de la cowdriose ont permis de caractériser, pour la première fois, les réponses T protectrices induites au cours de la vaccination. Ces résultats ont fait l'objet de trois publications dans des revues à bureau de lecture (cf. articles 4, 5 et 6 en annexe). L'intérêt de ces recherches pour le développement d'un vaccin sous-unitaire a conforté la position de leader du Cirad-emvt pour les aspects immunologie et vaccinologie au niveau européen. De 1995 à fin 1996, j'ai été rapporteur pour le volet « Vaccins contre la cowdriose » du projet européen d'action concertée sur les tiques et maladies transmises (DGXII).

- début 1997 à fin 2003: affecté au Cirad-emvt en Guadeloupe. Au cours de mon séjour, j'ai contribué à la mise sur pied d'une structure de recherche en immunologie cellulaire. L'adaptation, chez la chèvre, de méthodes non-radioactives d'analyse des réponses cellulaires (basées essentiellement sur la cytométrie en flux) a fait l'objet d'une publication dans la revue "Cytometry" (cf. article 7). Ces méthodes ont permis d'améliorer la connaissance générale des mécanismes immunitaires protecteurs induits chez la chèvre par le vaccin inactivé contre la cowdriose. Au cours de cette période, j'ai supervisé plusieurs projets en collaboration avec des équipes étrangères:

(a) avec l'équipe du Prof. K. Sumption du CTVM (Center for Tropical Veterinary Medicine, Université d'Edinburgh, Grande-Bretagne) sur la validation d'un vaccin vivant atténué contre la cowdriose produit dans des cellules de tiques.

(b) avec l'équipe du Prof. L. Babiuk du VIDO (Veterinary Infectious Diseases Organisation, Canada) sur l'évaluation d'oligos CpG comme adjuvant pour le vaccin inactivé contre la cowdriose.

(c) avec l'équipe du Dr D. Du Plessis de l'OVI (Onderstepoort Veterinary Institute, Afrique du Sud) sur le fractionnement de protéines par électrophorèse en flux continu.

(d) avec le CERVA (Centre d'Etudes en Recherche Vétérinaire et Agrochimiques, Bruxelles) et l'Université de Namur (Prof. JJ Letesson) en Belgique pour la mise au point sur modèle caprin de la technique de marquage intracellulaire de cytokines par cytométrie en flux.

Gestion de la recherche

- 2000/2001 : coordinateur d'un projet de recherche sur la cowdriose en collaboration avec l'OVI en Afrique du Sud. Titre: "Développement d'un vaccin recombinant contre la cowdriose ou (heartwater) des ruminants". Financement MAE/MNERT, programme franco-sud-africain de coopération en recherche scientifique et technique (PO 00-3608/SPE/PM/SUB).

Encadrement

- depuis juin 2000, encadrement (co-promoteur) de la thèse de doctorat en Sciences de Mlle I. Esteves, intitulée « Analyse des réponses T induites chez la chèvre par le vaccin inactivé contre la cowdriose des ruminants (infections à Ehrlichia ruminantium)». Soutenance prévue

février/mars 2004 à l'Université de Namur en Belgique (promoteur académique, Prof. JJ Letesson).

P. Totté, Esteves I, Gunter N, Martinez D, Bensaid A. 2002. Evaluation of several flow cytometric assays for the analysis of T-cell responses in goats. *Cytometry* 49(2); 49-55.

I. Esteves, K. Walravens, N. Vachiery, D. Martinez, J.J. Letesson, and P. Totté. 2004. Protective killed *Ehrlichia ruminantium* vaccine elicits IFN-γ responses by CD4+ and CD8+ T lymphocytes in goats. *Veterinary immunology and immunopathology* 98; 49-57.

I. Esteves, D. Martinez and P. Totté. Identification of *Ehrlichia ruminantium* (Gardel strain) IFN-γ inducing proteins after vaccination with a killed vaccine. Soumis à *Veterinary Microbiology*.

I. Esteves, N. Vachiéry, D. Martinez and P. Totté. Analysis of *Ehrlichia ruminantium*-specific T1/T2 responses during vaccination with a protective killed vaccines and challenge of goats. Soumis à *Parasite Immunology*.

- contribution sous forme d'articles scientifiques aux thèses de D. Martinez (1997, Faculty of Veterinary Medicine, Université d'Utrecht, Pays-bas) et N. Vachiéry (1998, Université Paris 7).

Travaux scientifiques

L'essentiel de ma contribution réside dans la caractérisation de mécanismes immunitaires protecteurs dans les infections à *Ehrlichia* (Ex *Cowdria*) ruminantium, agent causal de la cowdriose (ou "heartwater") des ruminants en Afrique sub-saharienne et dans la Caraïbe.

Au cours de ma thèse de doctorat, j'ai identifié pour la première fois deux facteurs endogènes capables d'inhiber le développement de ce pathogène intracellulaire dans sa cellule cible, la cellule endothéliale. Le premier, l'interféron alpha (IFN-α), est détecté dans la circulation sanguine des bovins qui survivent sans antibiothérapie à un challenge expérimental (article 1). De plus, in vitro, l'IFN-α recombinant diminue la quantité de corps infectieux relargués par les cellules endothéliales infectées (article 1). Cependant, le développement d'*E. ruminantium* n'est que partiellement inhibé même en présence de concentrations élevées d'IFN-α ce qui suggère l'intervention d'autres facteurs ou mécanismes dans le contrôle de l'infection. Un de ces facteurs pourrait être l'interféron gamma (IFN-γ), une cytokine produite entre autre par les lymphocytes T. En effet, l'IFN-γ inhibe complètement le développement d'*E. ruminantium* in vitro dans les cellules endothéliales a des concentrations non cytotoxiques (article 2). En revanche, l'infection par le pathogène diminue de façon spectaculaire l'expression des molécules de classe II du CMH induite par l'IFN-γ. Cet effet immunosuppresseur d'*E. ruminantium* a été confirmé et étendu aux molécules de classe I (cf. réf. 6 page 3). Ces résultats suggèrent fortement que ce pathogène intracellulaire strict a développé des mécanismes d'échappement à la reconnaissance par le système immunitaire de l'hôte. Les propriétés thérapeutiques de l'IFN-γ ont été confirmées in vivo dans un modèle murin de la cowdriose (article 3).

L'intérêt de l'IFN-γ pour le développement d'un vaccin réside dans le fait qu'il peut être produit par les lymphocytes T mémoires spécifiques du pathogène. Il restait néanmoins à démontrer que c'était effectivement le cas pour *E. ruminantium*. Mes travaux à l'ILRI pendant mon post-

doc ont permis de valider cette hypothèse et d'apporter pour la première fois des informations sur le type de réponses T associées à l'immunité protectrice contre la cowdriose chez le ruminant. La vaccination avec des bactéries inactivées et émulsifiées dans un adjuvant huileux confère aux bovins une immunité durable (article 4). Les lignées cellulaires spécifiques d'*E. ruminantium* dérivées à partir de ces animaux sont constituées pour plus de 95% de cellules T CD4+ (T auxiliaires). Ces lignées prolifèrent et produisent de l'IFN-γ en réponse aux antigènes du pathogène d'une manière dépendante des molécules de classe II du CMH (article 4). De plus, elles ont été générées 8 mois après la vaccination ce qui implique la présence de T mémoires. Ces lignées enrichies en CD4 reconnaissent de manière spécifique les protéines solubles et surtout membranaires d'*E. ruminantium* (article 5). Elles ont permis une première caractérisation du poids moléculaire des protéines solubles d'"*E. ruminantium* capables d'induire la production d'IFN-γ . Enfin, elles se sont révélées très sensibles puisque capables de détecter des protéines à des concentrations inférieures à 1 µg/ml (article 5).

Un article de synthèse sur les réponses immunitaires induites par *E. ruminantium* et les antigènes impliqués dans ces réponses est paru dans la revue "Parasitology Today" (article 6).

Mon séjour à l'ILRI n'a pas pu être prolongé pour différentes raisons et j'ai donc intégré l'équipe du Cirad-emvt travaillant sur la cowdriose et basée en Guadeloupe (Caraïbes). L'objectif principal était d'analyser les réponses cellulaires associées à l'immunité protectrice dans un modèle caprin. L'implication des lymphocytes T CD4 dans l'immunité induite par le vaccin tué a été confirmée (article 7). Cependant, plusieurs différences notables avec le modèle bovin existent. Premièrement, une activation spécifique des CD8 en réponse aux antigènes d'"*E. ruminantium* est observée. Deuxièmement, les adjuvants huileux utilisés induisent une forte activation spontanée des lymphocytes T caprins in vitro ce qui complique l'analyse des réponses spécifiques du pathogène. Enfin, il n'y a pas ou peu de prolifération active des cellules avec enrichissement d'une ou plusieurs sous-populations comme c'est le cas chez le bovin. Néanmoins, la capacité des CD4 et CD8 à produire de l'IFN-γ en réponse aux antigènes d'"*E. ruminantium* a été démontrée chez les chèvres immunisées (article 8). La contribution comparable des CD8 par rapport aux CD4 est assez inattendue dans la mesure ou les animaux ont été vaccinés avec des organismes tués. Cependant, il s'agit fort probablement d'un cas de coopération cellulaire CD4-CD8 puisque la production d'IFN-γ est entièrement bloquée par l'ajout d'anticorps dirigés contre les molécules de classe II du CMH. Enfin, la contribution d'antigènes d'"*E. ruminantium* présentés en association avec les molécules de classe I a également été mise en évidence dans deux cas sur trois (article 8).

Projet de recherche

Titre: Application de l'immunologie moderne au développement de vaccins contre les maladies vétérinaires tropicales.

Contexte scientifique: Dans le cadre de son programme en santé animale, le Cirad-emvt contribue à la mise au point de vaccins contre les grandes pathologies animales tropicales. Une approche génomique-transcriptomique-protéomique est actuellement poursuivie pour deux maladies d'origine bactérienne: la cowdriose (infection à *Ehrlichia ruminantium*) et la péripneumonie contagieuse bovine (PPCB, infection à *Mycoplasma mycoides*). Cette approche comporte deux volets: a) l'identification de gènes impliqués dans la virulence afin de développer des vaccins vivants atténués; b) l'identification de gènes impliqués dans la protection pour la mise au point de vaccins sous-unitaires. Dans les deux cas, une connaissance approfondie des mécanismes immunitaires induits au cours de l'infection est nécessaire. En effet, elle permet de développer des outils cellulaires appropriés pour la sélection d'antigènes et de gènes potentiellement intéressants d'un point de vue vaccinal. Enfin, elle est indispensable pour un choix judicieux des adjuvants et des systèmes de délivrance antigénique dans le cas de vaccins sous-unitaires.

D'un point de vue immunologique trois axes de recherche sont prioritaires: a) rôle des lymphocytes T; b) mécanismes d'échappement à la réponse immunitaire; c) processus inflammatoire.

Objectifs:

- caractérisation fine des réponses immunitaires impliquées dans la protection contre et dans la pathologie de la cowdriose et de la PPCB.
- développement de tests cellulaires immunologiques pour la sélection d'antigènes
- identification des gènes des pathogènes impliqués dans la pathologie et la protection.

Méthodologies:

a) analyse transcriptomique des interactions pathogènes-cellules (cellules dendritiques, macrophages et cellules endothéliales pour *E. ruminantium*) par micro-arrays en utilisant le « Ruminant Immune Gene Universal Array » (RIGUA) qui permet l'analyse de 516 gènes. Une analyse plus spécifique pourra être faite par RT-PCR quantitative et cytométrie en flux. Comparaison pathogènes virulents v/s atténués v/s inactivés.

b) utilisation de cellules dendritiques (CD) pour générer des lignées/clones enrichies en lymphocytes T spécifiques de MmmSC. Les CDs sont les seules cellules capables de présenter les antigènes à la fois aux lymphocytes T naïfs et mémoires. La comparaison (phénotypique et fonctionnelle) entre les lignées obtenues sur animaux naïfs d'une part, et sur animaux immunisés et éprouvés d'autre part, devrait permettre d'identifier le/les phénotype(s) associé(s) à la pathologie et à la protection.

c) mise en évidence directe in vivo du rôle des lymphocytes T par déplétion cellulaire au moyen d'anticorps et par transfert d'immunité sur bovins jumeaux monozygotes.

d) fractionnement des protéines par électrophorèse 2-D et transfert sur nitrocellulose. Criblage des spots à l'aide des lignées générées en b). D'autres tests développés en a) seront également utilisés (expression des molécules du CMH, cytokines, etc...).

e) analyse des spots par spectrométrie de masse et identification des gènes correspondants à partir des séquences complètes des génomes qui sont disponibles.

Article 1

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Production of Alpha Interferon in *Cowdria ruminantium*-Infected Cattle and Its Effect on Infected Endothelial Cell Cultures

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Cattle that resisted experimental heartwater infection caused by the rickettsia *Cowdria ruminantium* produced significant levels of circulating alpha interferon (IFN-α), whereas animals that died from heartwater did not. In vitro, recombinant bovine IFN-α was found to significantly reduce the yield of *Cowdria* organisms in bovine endothelial cells, but even at a high concentration (1,000 U/ml), IFN-α did not completely prevent the growth of *Cowdria* organisms in these cells. This limited inhibitory effect of IFN-α is in agreement with the in vivo situation where an infectious process has to take place to induce a protective immune response. Our results suggest that IFN-α produced in vivo in response to *Cowdria* infection may represent an efficient way to slow down the infection and allow the animal to mount a protective immune response. IFN-α is the first endogenously produced factor shown to have anti-*Cowdria* activity.

Cowdriosis, or heartwater, is a tick-borne disease of wild and domestic ruminants caused by the rickettsia *Cowdria ruminantium*. The disease is endemic in Sub-Saharan Africa, where it represents a major obstacle to the upgrading of cattle (20). Moreover, cowdriosis has been found in the Caribbean, and the risk of introduction of the disease onto the American mainland exists (21). In infected ruminants, *Cowdria* organisms replicate within membrane-bound vacuoles in the cytoplasm of endothelial cells of blood vessels (12). Without appropriate antibiotic therapy, the disease is generally fatal in susceptible animals, which develop respiratory, digestive, and nervous disorders (20). Methods of vaccination against heartwater exist (e.g., infection of the animals with virulent sheep blood vaccine followed by tetracycline treatment of the clinical reaction), but their application is risky, time consuming, and laborious (10). Other means of control are needed, and from this perspective, a better understanding of the mechanisms involved in the protective immune response against *Cowdria* organisms is of major importance.

Interferons (IFNs) have been shown to play an important role in the resistance against obligate intracellular parasites including members of the order *Rickettsiales* (3). The capacity of IFNs to induce an antirickettsial state in macrophages but also in nonprofessional phagocytes such as fibroblasts (13) and epithelial (16) and endothelial (23) cells is well established. The mechanisms underlying these effects are not yet fully understood. Stimulation of the microbicidal activity of macrophages by regulating their oxygen metabolism (9), reduction in the level of tryptophan in fibroblast and epithelial cells (2, 15), and synthesis of reactive nitrogen intermediates from L-arginine (11) are among the possible causes described to date. IFNs are also involved in the growth and activation of lymphocytes in response to infection (4).

As far as *Cowdria* infections are concerned, very little concerning the possible role of IFNs is known. We have shown previously (18) a positive correlation between resistance and presence of antiviral activity in the plasma of experimentally *Cowdria*-infected cattle. In this report, we characterize this activity as alpha interferon (IFN-α) and demonstrate its inhibitory effect on the growth of *C. ruminantium* in bovine endothelial cells.

MATERIALS AND METHODS

Experimental animals. The in vivo part of the study was carried out in Harare, Zimbabwe, at the Veterinary Research Laboratories. Eight Frisian cows (between 1 and 2 years old) were used for the experiment. Seven animals were each inoculated intravenously with 5 ml of *Cowdria* (Palm River isolate)-infected deep-frozen sheep blood (10). One animal was kept as an uninfected control. Of the infected animals, two were treated with oxytetracycline (Terramycin; Pfizer Inc.; 5 mg/kg of body weight subcutaneously) on the second day of fever to ensure survival. The rectal temperature of the animals was monitored daily, blood samples were collected in heparin and centrifuged at 300 × *g* for 30 min, and the plasma was frozen at −70°C. The animals that survived the infection each received 5 ml of *Cowdria*-infected sheep blood as a homologous challenge 36 days later.

Competitive ELISA. *Cowdria* antigens for the competitive enzyme-linked immunosorbent assay (competitive ELISA) were prepared from rickettsiae cultivated in bovine umbilical endothelial cells as described previously (7). The competitive ELISA used an immunoglobulin G3 monoclonal antibody (4F10B4), which reacts with epitopes on a 32-kDa *Cowdria* surface protein (Cr32), and was performed as described previously (5) but with minor modifications (8). Briefly, 96-well polystyrene plates (Nunc) were coated overnight with 6 μg of sonicated *Cowdria* antigen per ml in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plates were washed three times with

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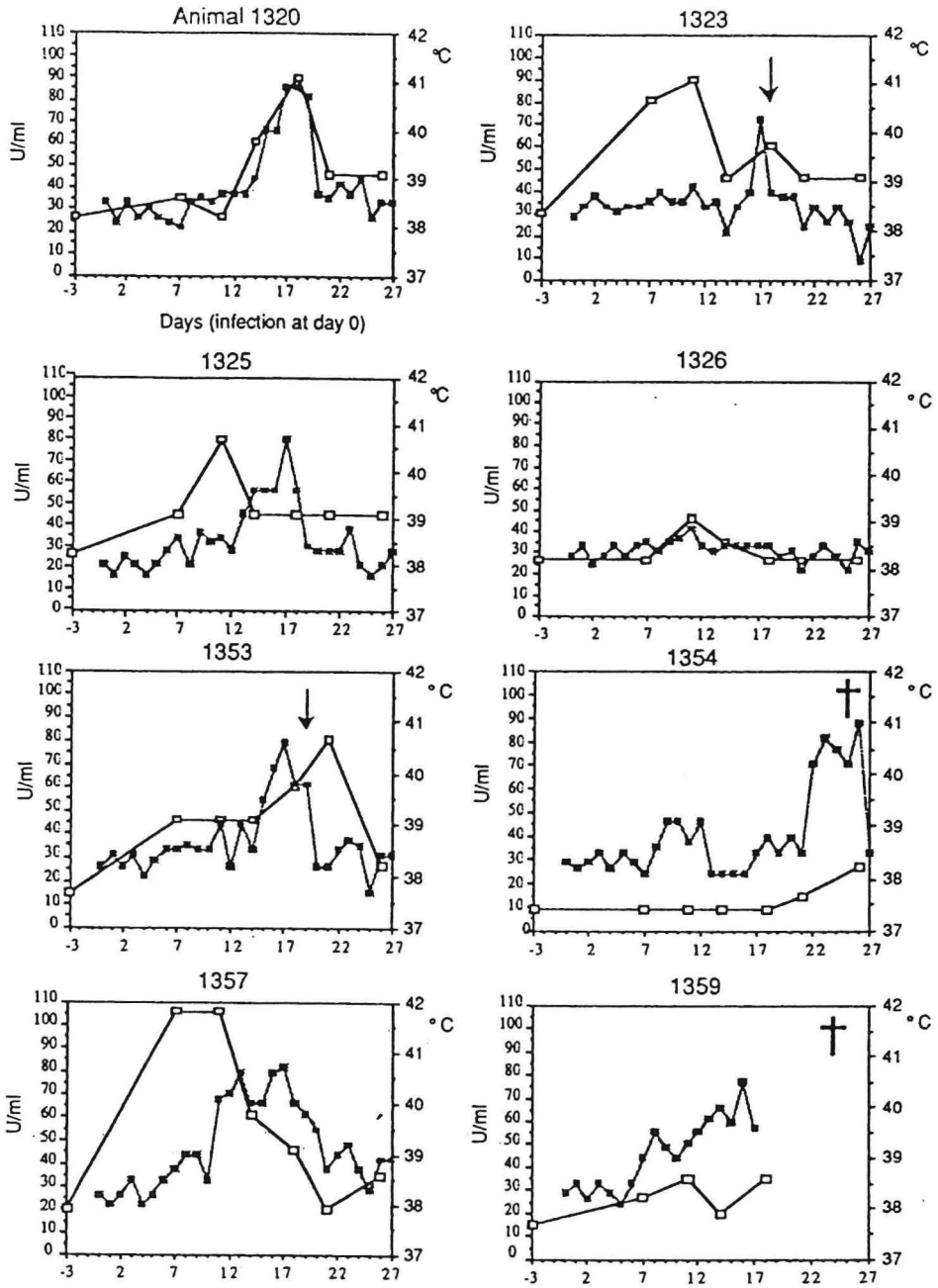

FIG. 1. Circulating antiviral activity (□) and rectal temperature (▩) of *C. ruminantium*-infected cattle. Animal 1326 was included as a noninfected control; animals 1323 and 1353 were treated with terramycin (arrow); animals 1354 and 1359 died of heartwater (†).

phosphate-buffered saline (PBS; pH 7.2). Test sera (1:50 dilution) were applied simultaneously with monoclonal antibody 4F10B4 (final dilution, 10 μg/ml), both of which were diluted in PBS containing 3% milk and 0.05% Tween 20 and incubated at 37°C for 1 h. After the plates were washed, peroxidase-labeled rabbit anti-mouse immunoglobulin (Dakopatts), at a 1:750 dilution, was added, and the plates were incubated at 37°C for another hour. After the plates were washed, 100 μl of ABTS (Sigma Chemical Co., St. Louis, Mo.) substrate solution was dispensed into each well, and the optical density at 405 nm was measured after 30 min of incubation at room temperature.

Cell culture and *Cowdria* cultivation in vitro. Bovine endothelial cells from the microvasculature (BME cells) of brain adrenocortex (provided by G. Tarone, Department of Genetics, Biology, and Medical Chemistry, University of Torino, Torino, Italy) were used for the in vitro experiments. Since *Cowdria* stabilates of the Palm River isolate used for in vivo studies were not yet adapted for in vitro culture, we used the Senegal isolate of *C. ruminantium*, which has been described earlier (6). The cultivation of *C. ruminantium* (Senegal) in BME cells was done as previously described for bovine umbilical endothelial cells (7). Briefly, BME cells were grown to confluence in a 75-cm^2 tissue culture flask (Nunc). The cells

TABLE 1. Characterization of circulating antiviral activity by using antibodies against BoIFN-α

Antibodies	Antiviral activity (U/ml) of animal no.:					
	1320 at day 18 p.i.[a]	1323 at day 11 p.i.	1325 at day 11 p.i.	1353 at day 21 p.i.	1357 at:	
					day 6 p.i.	day 11 p.i.
Absent	90	90	80	80	140	140
Present	10	10	ND[b]	ND	46	35

[a] p.i., postinfection.
[b] ND, not done.

were infected with sucrose-phosphate-glutamate (SPG) buffer (1)-cryopreserved *Cowdria* stabilates diluted in Glasgow minimum essential medium (GIBCO) supplemented with tryptose phosphate broth (GIBCO) at 2.9 g/liter, penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (20 mM; pH 7.0 to 7.2), L-glutamine (2 mM), and 10% fetal calf serum (GIBCO/BRL). The cells were incubated without shaking in a 37°C incubator with 5% CO_2. When more than 70% of the cells were lysed, the culture supernatant was centrifuged for 15 min at 15,000 × *g*; the pellet obtained from 2 ml of supernatant was resuspended in 1 ml of SPG buffer and snap frozen in liquid nitrogen. *Cowdria* stabilates at passages 9 to 11 (average interval of 10 days between each passage) and BME cells at passage 20 were used in this study.

IFN, IFN assay, and anti-IFN antibodies. Recombinant bovine IFN-αC (rBoIFN-αC; specific activity, 2×10^5 U/mg) was cloned and expressed in *Escherichia coli* (provided by A. Shafferman, Israel Institute for Biological Research, Ness-Ziona). The antiviral activity of the samples was estimated on Madin-Darby bovine kidney (MDBK) cells by using the classical test of reduction of the cytopathogenicity of vesicular stomatitis virus (17). The antiviral activity is expressed in laboratory units; no international standards are available as references for bovine IFNs. One unit of antiviral activity is defined as the reciprocal of the dilution that gives 50% protection against viral challenge under standard conditions. The antiviral activity was characterized by using neutralizing antibodies (rabbit polyclonal antibodies) to BoIFN-α (provided by R. Steiger, CIBA-GEIGY); a 10-fold dilution of the stock neutralizes 10^4 U of BoIFN-αC. These antibodies were reported by the manufacturer not to cross-react with any other known bovine cytokine.

Anti-*Cowdria* activity of BoIFN-αC. The effect of BoIFN-αC on the infectious yield of *C. ruminantium* was studied by titrating the infectivity of supernatants from *Cowdria*-infected (in the presence or absence of IFN) BME cells by using a 50% tissue culture lethal dose ($TCLD_{50}$) test (see below). Supernatant freshly collected from a 75-cm^2 flask, showing 70% or more lysis due to *Cowdria* infection, was used as the inoculum. The infected supernatant was centrifuged for 5 min at 300 × *g* to remove cell debris and diluted twofold in complete Glasgow minimum essential medium (see "Cell culture and *Cowdria* cultivation in vitro"). Five hundred microliters of the diluted supernatant was added to confluent BME cells in a 24-well plate at 37°C and under 5% CO_2. After 24 h of incubation, the medium was renewed. At day 5 postinfection, 500 μl of fresh medium was added to the wells. BoIFN-αC was added every day starting on day 0 and until day 2 postinfection. Control cells, IFN treated but not infected, were included in each experiment. To ascertain the involvement of IFN-α, anti-BoIFN-α antibodies were incubated together with IFN (20 min at 37°C) before being added to the cells instead of IFN alone. The progress of the infection was monitored daily by light microscopy. Supernatants were collected every day starting at day 7 at a time when lysis of the cells shows up clearly in infected untreated wells and until complete destruction of the monolayer at day 10. For each IFN dilution, one well was kept until day 13 to study the progression of *Cowdria* infection under IFN treatment. Each supernatant was centrifuged at 15,000 × *g* for 15 min, and the pellet was resuspended in 100 μl of SPG buffer before being snap frozen in liquid nitrogen. The infectivity of these supernatants was measured by using the $TCLD_{50}$ assay as described below.

$TCLD_{50}$ assay for titration of *C. ruminantium*. BME cells were grown to confluence in 96-well plates (Nunc). *Cowdria* samples cryopreserved in SPG buffer were thawed at 37°C, serially diluted twofold in complete medium to a final volume of 100 μl per well, and added to the cells. The medium was replaced 24 h after infection, and subsequently, half of it was renewed every 4 days. The plates were incubated for 15 days in a 37°C incubator with 5% CO_2. Negative controls were included on every test plate to detect eventual lysis due to reasons other than *Cowdria* infection. After incubation, the cells were fixed in formaldehyde (35%) for 30 min and stained with crystal violet. The end point (50% lysis) was determined by light microscopy or by measuring the A_{540}. The $TCLD_{50}$ of the sample is the reciprocal of the dilution that gives 50% lysis of the cells after 15 days of incubation. The $TCLD_{50}$ test measures the infectivity of the samples, which depends on the concentration of *Cowdria* elementary bodies.

RESULTS

Induction of IFN-α in cattle experimentally infected with *C. ruminantium*. Two of the seven infected animals (no. 1354 and 1359) died of heartwater as confirmed by demonstration of rickettsial colonies in brain crush smears. Five of them (no. 1320, 1323, 1325, 1353, and 1357) survived the infection and were subsequently fully immune to homologous challenge. In the plasma of the survivors, a significant increase of antiviral activity was observed (Fig. 1). On the other hand, both animals that died did not show a significant increase in circulatory antiviral activity (Fig. 1). For all animals tested, the activity was ascribed to IFN-α by using neutralizing antibodies with titers ranging between 80 and 105 U/ml (Table 1).

Serology. Of the seven calves inoculated with the Palm River isolate, antibodies to the Cr32 protein of *C. ruminantium* were detected (Fig. 2) in four surviving animals (no. 1320, 1323, 1325, and 1353). Animal 1353 did not seroconvert after the primary infection but developed antibodies after the challenge. Seroconversion could not be detected in animal 1357, neither after the primary infection nor after challenge, although it proved solidly immune (poor handling and/or storage of samples?). No antibodies to *C. ruminantium* were detected in negative control calf 1326. Finally, antibodies were not de-

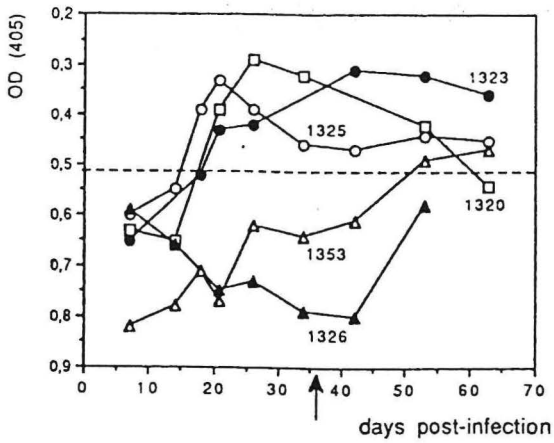

FIG. 2. Competition between antibodies in plasma from *C. ruminantium*-infected calves and anti-*Cowdria* monoclonal antibody 4F10B4 in a competitive ELISA. The mean optical density of 0.70 of seven nonimmune serum samples (animal 1326) minus 2 standard deviation units of 0.091 provided the dashed baseline of 0.518. Animals were challenged on day 36 (arrow). OD (405), optical density at 405 nm.

tected in animals 1354 and 1359, which both succumbed to heartwater.

Effect of BoIFN-α on the infectious yield of *C. ruminantium* in BME cells. The development of *C. ruminantium* (observed by light microscopy) in BME cells in the absence of IFN was similar to observations reported previously for endothelial cells isolated from bovine umbilical cord (7). Nonfusing colonies containing *Cowdria* reticulate bodies were detected in the cytoplasm of cells from day 4 after infection. Cell lysis (with release of infectious elementary bodies) occurred at day 5 and increased progressively until complete destruction of the monolayer had occurred by days 10 to 11 postinfection. The infectious yield of *Cowdria* organisms (as measured by the infectivity of supernatants) reached a peak at day 9, which corresponded to ±80% cell lysis, and then slowly decreased (Fig. 3).

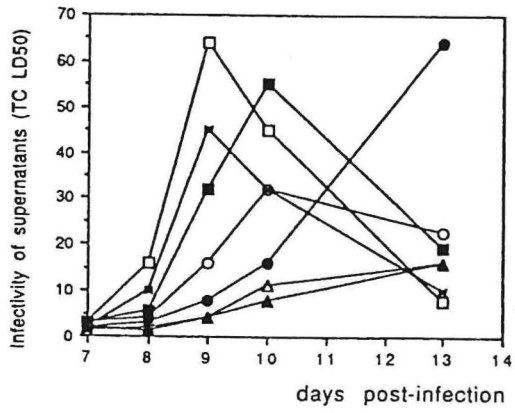

FIG. 3. Inhibitory effect of rBoIFN-α on the infectious yield of *C. ruminantium* in BME cells. IFN at doses of 0 (□), 1 (■), 10 (○), 100 (●), 500 (△), and 1,000 (▲) U/ml was studied. The concentration of 100 U of IFN per ml was chosen (because it has a significant inhibitory effect on *Cowdria* yield and to limit antibody consumption) for anti-rBoIFN-α antibody studies (*). The experiment was run in triplicate with a maximal standard deviation of ±11.0.

BoIFN-α reduced the yield of *Cowdria* infectious organisms in a dose-dependent way (Fig. 3). One hundred units of IFN provoked a 10-fold reduction of the yield at day 9. However, even at high concentrations (e.g., 1,000 U/ml), IFN never completely prevented the growth of *Cowdria* organisms in BME cells (*Cowdria* colonies were always visible in the cytoplasm of some cells) but delayed the progression of the infection. Reduction of the *Cowdria* infectious yield was counteracted by antibodies against rBoIFN-α (Fig. 3), confirming that BoIFN-α itself is responsible for the anti-*Cowdria* effect. BoIFN-α alone or in the presence of *Cowdria* organisms was not cytotoxic for the cells.

DISCUSSION

We found a positive correlation between resistance to *Cowdria* infection and the presence of a significant antiviral activity in the plasma of infected cattle. It is interesting to note that only in the case of animal 1353, which failed to produce anti-*Cowdria* antibodies after the first infection (suggesting that the animal would have died without antibiotic therapy), did the peak of antiviral activity appear very late (e.g., 5 days after the onset of fever). For all of the animals tested, the antiviral agent was characterized as IFN-α. In vivo titers ranged between 80 and 100 U/ml, depending on the animal. Although these titers are not very high, they are in the order of magnitude observed in animals intramuscularly injected with 10^5 to 10^6 U of IFN-α per kg and fully protected against experimental viral infection (14). It should also be pointed out that blood samples were collected from infected cattle at such intervals that the peak of circulating IFN could have been missed (e.g., IFN-α has a lifetime in the circulation of 24 h [22]). Such an amount (80 to 100 U/ml) of IFN-α was found to inhibit the in vitro development of *Cowdria* organisms in bovine endothelial cells. This inhibitory effect was dependent on the dose of IFN used and was completely neutralized by anti-BoIFN-α antibodies. The mechanisms underlying the anti-*Cowdria* effect of IFN-α are not yet known. The addition of tryptophan to the culture medium did not have any effect (19); the possible involvement of L-arginine in our model is under study.

Interestingly, even at very high concentrations (e.g., 1,000 U/ml), rBoIFN-α never completely prevented *Cowdria* growth but significantly reduced the number of infected cells. This is in agreement with the in vivo situation where an infectious process must take place to induce a protective immune response (e.g., moreover, if infected animals receive a high dose of antibiotics before the rise in temperature, they will survive the infection but will not be immune to subsequent homologous challenge). Therefore, IFN-α produced in vivo in response to *Cowdria* infection may represent a way to slow down the infection and allow the animal to mount a protective immune response. It is generally accepted that protective immunity in heartwater infections is predominantly cell mediated, but the exact immune effector mechanisms are not known. Our results indicate that IFN-α may play an important role in the resistance of cattle to *Cowdria* infections. However, it is realized that this study is based on only a small number of animals and that our conclusion that antiviral activity is directly linked with death or survival of these cattle should be confirmed by using a larger number of heartwater-infected animals. Also, a comparison of different *Cowdria* isolates, including Palm River, for their sensitivity to IFN-α in vitro should be made.

IFN-α is the first endogenously produced substance with anti-*Cowdria* properties in vitro. We have now undertaken

studies in vivo to evaluate the efficacy of recombinant IFN treatment against *Cowdria* infection. Further studies are also in progress to study the role of IFN-γ as well as other cytokines in *Cowdria* infections.

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Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells

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Abstract

Recombinant bovine IFNγ is a potent inhibitor of *Cowdria ruminantium* growth in vitro irrespective of the rickettsial stock, or the origin of the endothelial cells. These results suggest an important role for IFNγ in protective immune responses against *C. ruminantium* infections. Here we also show that IFNγ can induce the expression of MHC class II molecules on the surface of endothelial cells. However, treatment of endothelial cells with IFNγ following infection with *Cowdria* fails to induce MHC class II expression. The implications of this pathogen-specific effect on class II expression by endothelial cells with regard to its recognition by the host immune system are discussed.

Keywords: Cowdria ruminantium; IFNγ; MHC class II; endothelial cells

1. Introduction

Cowdriosis or heartwater is a tick-borne disease of wild and domestic ruminants caused by the obligate intracellular rickettsia *Cowdria ruminantium* (Cowdry, 1925) that

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is endemic in subsaharan Africa and in the West Indies (Uilenberg, 1983). Although a degree of innate resistance against cowdriosis exists among indigenous ruminants, the disease becomes a major problem when exotic breeds are introduced (Provost and Bezuidenhout, 1987). Also, because of the proximity of the West Indies to the American mainland, cowdriosis is a potential threat to the American livestock market (Uilenberg, 1982; Barre et al., 1987).

Animals that survive *Cowdria* infection are immune to homologous challenge but the mechanisms involved in protective immunity against *C. ruminantium* are poorly understood. Preliminary studies suggest that the nature of protective immunity is largely cell-mediated (Uilenberg, 1983). The transfer of Lyt-2^+ T lymphocytes from immunized mice protects susceptible mice against *C. ruminantium* infection (Du Plessis et al., 1991). However, the exact mechanisms of protection effected by immune Lyt-2^+ cells remain to be defined. We have shown recently (Totté et al., 1994) that interferon alpha (IFNα), which can be produced by activated T cells (Ho, 1984), is induced in cattle that naturally (without antibiotic treatment) resist an experimental infection with *C. ruminantium*. The growth of the pathogen in vitro within bovine vascular endothelial cells (one of the target cells of *C. ruminantium* in vivo) is significantly retarded by recombinant bovine IFNα, but is not completely blocked (Totté et al., 1994), suggesting that other mechanisms are necessary for the control of the infection. A possible candidate is interferon gamma (IFNγ), which is also produced by activated T lymphocytes and has been shown to play an important role in the resistance against several other rickettsias (Byrne and Turco, 1988). Recombinant IFNγ inhibits the in vitro growth of *Rickettsia prowazekii* (Turco and Wrinkler, 1983), *R. tsutsugamushi* Gilliam strain (Hanson, 1991a), *Chlamydia psittaci* (Byrne et al., 1986), *Chlamydia trachomatis* (Shemer and Sarov, 1988) and *Ehrlichia risticii* (Park and Rikihisa, 1991). A protective role for endogenous IFNγ in vivo has been demonstrated for *R. conorii* (Li et al., 1987) and *Chlamydia trachomatis* (Williams et al., 1988; Rank et al., 1992). Another role for IFNγ is in the induction of major histocompatibility complex (MHC) class II expression in a variety of cells including bovine endothelial cells (Coutinho et al., 1991). The primary function of class II MHC molecules is to present foreign antigen-derived peptides to T cells (Hunt et al., 1992) resulting in the initiation of an immune response.

In this report, we show that recombinant bovine IFNγ completely prevents the multiplication of several stocks of *C. ruminantium* in bovine and caprine vascular endothelial cells in vitro (part of these results have been published in the Proceedings of the Second Biennial Meeting of the Society of Tropical Veterinary Medicine — STVM 93 — held in Guadeloupe, French West Indies, 2–6 February 1993) (Totté et al., 1993). Furthermore, we describe altered cell surface expression of MHC class II molecules by these cells following infection by *C. ruminantium* and IFNγ treatment.

2. Materials and methods

2.1. Cell cultures

One caprine and two bovine endothelial cell lines were used in this study. Bovine endothelial cells isolated from microvessels of brain cortex (BME) were kindly provided

by Dr. G. Tarone (University of Torino, Italy). Bovine endothelial cells from umbilical cord arteries (BUEC) were a kind gift of Dr. F. Jongejan (University of Utrecht, the Netherlands). Caprine endothelial cells (CJE) were isolated from the jugular vein according to established procedures (Schwartz et al., 1991). These cells are positive for factor VIII production as shown by immunofluorescence, (data not shown), indicating that they are of endothelial origin. All these cell lines are fully permissive to *C. ruminantium* growth. All cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum (Gibco), penicillin (100 IU ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and L-glutamine (2 mM).

2.2. *Cowdria stocks and in vitro culture*

Three different stocks of *C. ruminantium* were used in this study, the Senegal stock (Jongejan et al., 1988), the Welgevonden stock from South Africa (Du Plessis, 1985) and the Gardel stock from Guadeloupe (Uilenberg et al., 1985). All these stocks were kept as stabilates of in vitro infected endothelial cells cryopreserved in sucrose–phosphate–glutamate (SPG) buffer (Bovarnick et al., 1950). Culture of *C. ruminantium* in endothelial cells was carried out as previously described (Totté et al., 1994). Briefly, endothelial cells were grown to confluency in 75 cm^2 tissue culture flasks (Nunc). Cells were infected using SPG-cryopreserved stabilates of *C. ruminantium* diluted in 'complete medium' composed of Glasgow minimum essential medium (GMEM, Gibco) supplemented with tryptose phosphate broth (Gibco) at 2.9 g l, penicillin (100 IU ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$); hepes (20 mM, pH 7.0–7.2), L-glutamine (2 mM) and 10% fetal calf serum (Gibco). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. *C. ruminantium* replicates in intracytoplasmic vacuoles, giving rise to large colonies (morulae), which can be easily observed under a light microscope (inversed microscope, Leitz Diavert) or after staining of the cells with Giemsa or Diff-quick (Baxter). When more than 70% of the cells were lysed by the rickettsia, the culture supernatant was centrifuged for 15 min at 15 000 g. The pellet obtained from 2 ml of supernatant was resuspended in 1 ml SPG buffer before snap freezing in liquid nitrogen.

2.3. *One-step growth — yield assay for C. ruminantium*

We have studied the effect of recombinant bovine IFNγ on the infectious yield of *C. ruminantium* from endothelial cells infected in vitro. This was done by titrating the infectivity of supernatants from endothelial cells infected with *Cowdria* in the presence or absence of IFNγ. Endothelial cells were grown to confluency in 24-well plates (Nunc) prior to infection with *Cowdria*-infected supernatant (1 ml well). Infected supernatant was prepared from a culture showing 70–80% cell lysis owing to *C. ruminantium* and diluted twofold in fresh complete medium prior to use. Triplicates wells were treated with medium alone (control) or with various concentrations of IFNγ at different time intervals. The medium was replaced 24 h after each treatment. In order to confirm the involvement of IFNγ, the experiment was repeated in the presence of neutralising antibodies for bovine IFNγ. The progress of the infection was followed daily by light microscopy. All supernatants were collected 9 days after infection, when

control wells (infected but not treated) showed 70–80% lysis owing to the rickettsia. Each supernatant was centrifuged at 15 000 g for 15 min and the pellet resuspended in 100 ml SPG before snap freezing in liquid nitrogen. The infectivity of these supernatants was measured by a TCLD50 assay adapted for *C. ruminantium* (Totté et al., 1994). Results are expressed as % inhibition of *Cowdria* yield (± standard deviation) compared with the control.

2.4. IFN, IFN assay, and anti-IFN antibodies

Recombinant bovine interferon gamma (rBoIFNγ, specific activity 2×10^6 U mg^{-1}) produced in *E. Coli* and neutralizing antibodies to BoIFNγ were kindly donated by Dr. R. Steiger (CIBA-GEIGY). The titer of rBoIFNγ was regularly measured by the classical test of reduction of the cytopathogenicity of vesicular stomatitis virus (Stewart, 1979) on Madin Darby bovine kidney cells (MDBK). The antiviral activity was expressed in laboratory units; no international reference standards are available for bovine IFNs. One unit of antiviral activity is defined as the reciprocal of the dilution that gives 50% protection against viral challenge under standard conditions. In our test, a tenfold dilution of anti-BoIFNγ antibodies was shown to neutralize 1.10^3 U of rBoIFNγ.

2.5. Flow cytometry analysis of endothelial cell surface class II molecules

BUE and CJE cells were grown and infected with the Gardel stock of *C. ruminantium* as described above. A total of 5 U ml^{-1} of recombinant IFNγ were added to each culture including non-infected samples at 4 and 24 h after infection. After 72 h, endothelial cell layers were gently trypsinized and washed in RPMI medium containing 10% fetal calf serum. Each sample was resuspended in 100 μl of FACS medium (RPMI 1640, 2% normal horse serum and 0.02% sodium azide) and divided into two aliquots where a control mAb (VC13) or an anti-MHC class II mAb (VC9) were added at a final dilution of 1/1000. After 45 min incubation, the cells were washed three times and resuspended in 50 μl of FACS medium containing FITC-labelled goat anti-mouse Ig (Caltag, San Francisco, CA) diluted 1/100. The cells were further incubated for 45 min and washed three times before analysis on a fluorescence activated cell sorter (FACSort apparatus, Becton Dickinson, San Jose, CA). Monoclonal antibody VC13 specifically recognizes BoCD1 molecules (N.D. MacHugh, personal communication, 1995). The specificity of mab VC9 for MHC class II molecules was determined by tissue distribution and immunoprecipitation studies and by its reactivity to previously described L-cell transfectants (Ballingall et al., 1995) as shown in Fig. 1.

3. Results

The effect of rBoIFNγ on the yield of *C. ruminantium* infectious particles from vascular endothelial cells was studied in vitro. rBoIFNγ reduced the yield of infectious *C. ruminantium* (Senegal stock) from BME cells in a dose-dependent manner (Fig. 2). Although pretreatment of BME cells with rBoIFNγ 24 h prior to infection resulted in

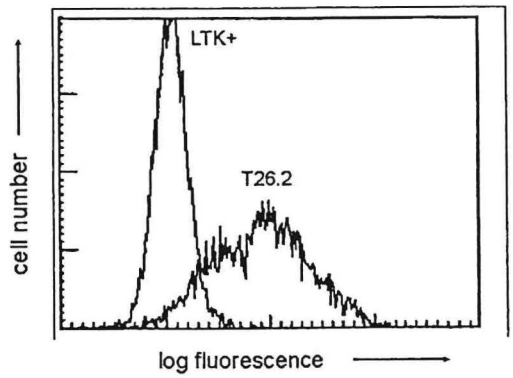

Fig. 1. The MHC class II specificity of the mAb VC9 was determined by FACS analysis of MHC class II-expressing L-cell transfectant T26.2 and control thymidine kinase transfected line LTK+. Cells were labelled with the mAb VC9 followed by FITC-IgG antimouse Ig as a second stage.

significant inhibition of *Cowdria* growth, rBoIFNγ was more effective when added after adsorption of *C. ruminantium* (e.g. 24 h after infection; Fig. 2). Under these conditions, 100% inhibition of *C. ruminantium* yield was achieved with as little as 0.5 U ml^{-1} of rBoIFNγ (Fig. 2). The inhibitory effect was completely reversed by addition of rBoIFNγ-specific antibodies to the medium (Fig. 2). Inhibition was unaffected by the addition of 100 U ml^{-1} of polymyxin B (a known chelator of bacterial endotoxins; not shown). Although 100% reduction of the *Cowdria* infectious yield was observed 9 days postinfection, when cells were treated with up to 5 U ml^{-1} of rBoIFNγ, colonies of *C. ruminantium* were still visible in the cytoplasm of some cells. Therefore the progression of the infection was delayed by IFNγ but not completely prevented. However, when the

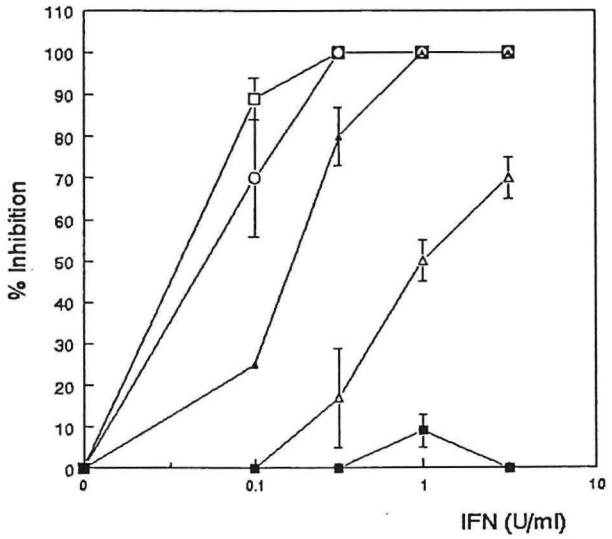

Fig. 2. Inhibition by rBoIFNγ of *C. ruminantium* (Senegal) infectious yield from BME cells. rBoIFNγ was added to the medium at different concentrations and time intervals: 24 h before infection (-△-); at day 0 (-▲-); at day 1 (-○-) and at days 0 and 1 (-□-). Some wells received rBoIFNγ at days 0 and 1 together with anti-rBoIFNγ (-■-). Data are mean values (± standard deviation) of four experiments.

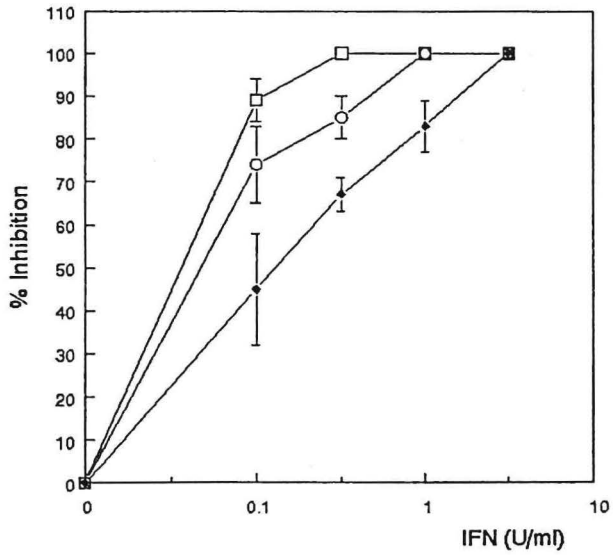

Fig. 3. rBoIFNγ-mediated inhibition of *C. ruminantium* (Senegal) infectious yield in different types of vascular endothelial cells. BME (-□-), BUE (-○-) and CJE (-♦-) cells were treated with various concentrations of rBoIFNγ at days 0 and 1 postinfection. Data are mean values (± standard deviation) of two experiments.

cells were treated with 10 U ml^{-1} of rBoIFNγ at days 0 and 1, colonies of *C. ruminantium* were not observed for up to 30 days postinfection (not shown). Cytotoxicity of rBoIFNγ for uninfected or *C. ruminantium* infected BME and BUE cells was observed only when 50 U ml^{-1} or more were added to the medium for three consecutive days.

As shown in Fig. 3, bovine endothelial cells are sensitive to the anti-*cowdria* effect of rBoIFNγ regardless their tissue of origin. Indeed, microvasculature (BME) or macrovasculature (BUE) endothelial cells became equally non-permissive to *C. ruminantium* replication after rBoIFNγ treatment (Fig. 3). Caprine endothelial cells from jugular veins (CJE) were also sensitive to the anti-*cowdria* effect of rBoIFNγ (Fig. 3).

The growth of three stocks of *C. ruminantium* originating from different geographical locations was found to be efficiently inhibited by IFNγ in BUE cells (Fig. 4). Although the *C. ruminantium* stocks used have been shown previously to be antigenically different in cross-immunity trials (15), our results demonstrate a dose-dependent inhibition of *Cowdria* growth in vitro irrespective of the stock used.

The endothelial cells used in this study, whether infected with *Cowdria* or not, did not constitutively express cell surface MHC class II molecules (Fig. 5a and Fig. 5b). Treatment with 5 U ml^{-1} of rBoIFNγ induced MHC class II expression on both BUE and CJE cells. Indeed, 55% and 46% of treated BUE and CJE cells, respectively, were positive for the mAb VC9 (Fig. 5c). In contrast, a significant inhibition of the expression of MHC class II molecules was observed on the surface of cells treated with IFNγ with only 10% and 7% of BUE and CJE cells, respectively, expressing the antigens after infection with *C. ruminantium* (Fig. 5d).

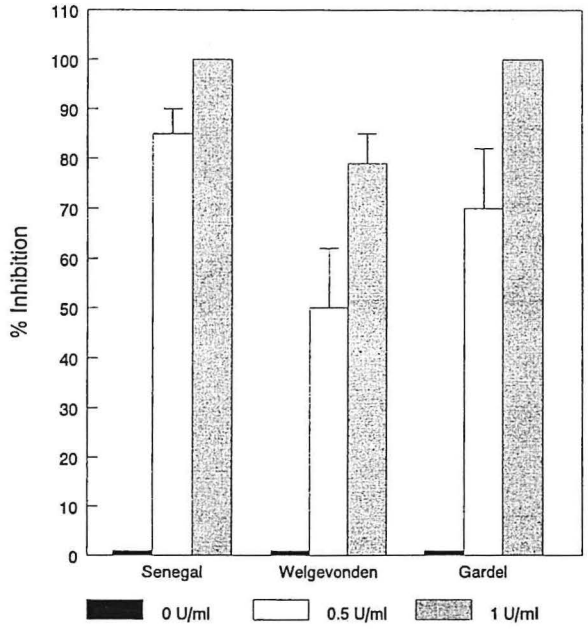

Fig. 4. Susceptibility of three different strains of *C. ruminantium* to the inhibitory effect of rBoIFNγ. BUE cells were treated with rBoIFNγ at days 0 and 1 postinfection. Data are expressed as mean values (± standard deviation) of triplicates.

4. Discussion

We have established that rBoIFNγ is a potent inhibitor of the growth of *C. ruminantium* in vascular endothelial cells in vitro. The growth of *C. ruminantium* in these cells is significantly inhibited at very low concentrations of rBoIFNγ, compared with that shown for other rickettsias in other cells. In our model, 10 U ml^{-1} of rBoIFNγ is capable of preventing the formation of *C. ruminantium* colonies, whereas 100 U ml^{-1} of human recombinant IFNγ are required to prevent infection of human epithelial cells by *Chlamydia trachomatis* (Shemer and Sarov, 1985). This may reflect differences between species and the type of cells used. We have previously demonstrated that bovine endothelial cells are ten to 20-fold more sensitive to the antiviral activity of bovine IFNs compared with bovine epithelial cells (Totté et al., 1993).

It is also apparent from this study that rBoIFNγ acts on the host cells to render them refractory to *C. ruminantium* replication rather than directly on the extracellular organism. Indeed, treatment of the cells with IFNγ at a time when extracellular *Cowdria* has been removed is more efficient than when simultaneous treatment and infection is performed. The formation of *C. ruminantium* colonies is prevented in vascular endothelial cells treated with rBoIFNγ, but the exact stage of the developmental cycle of *C. ruminantium* on which rBoIFNγ acts is not known. The inhibitory effect of rBoIFNγ on the infectious yield of *C. ruminantium* cannot be explained by specific lysis of infected cells (as it was shown for *Chlamydia trachomatis* (Byrne et al., 1988), *R. prowazekii*, (Turco and Wrinkler, 1984), and *R. tsutsugamushi* (Hanson, 1991a)) since no cytotoxic effect was detected at concentrations of rBoIFNγ that prevented the formation of *C.*

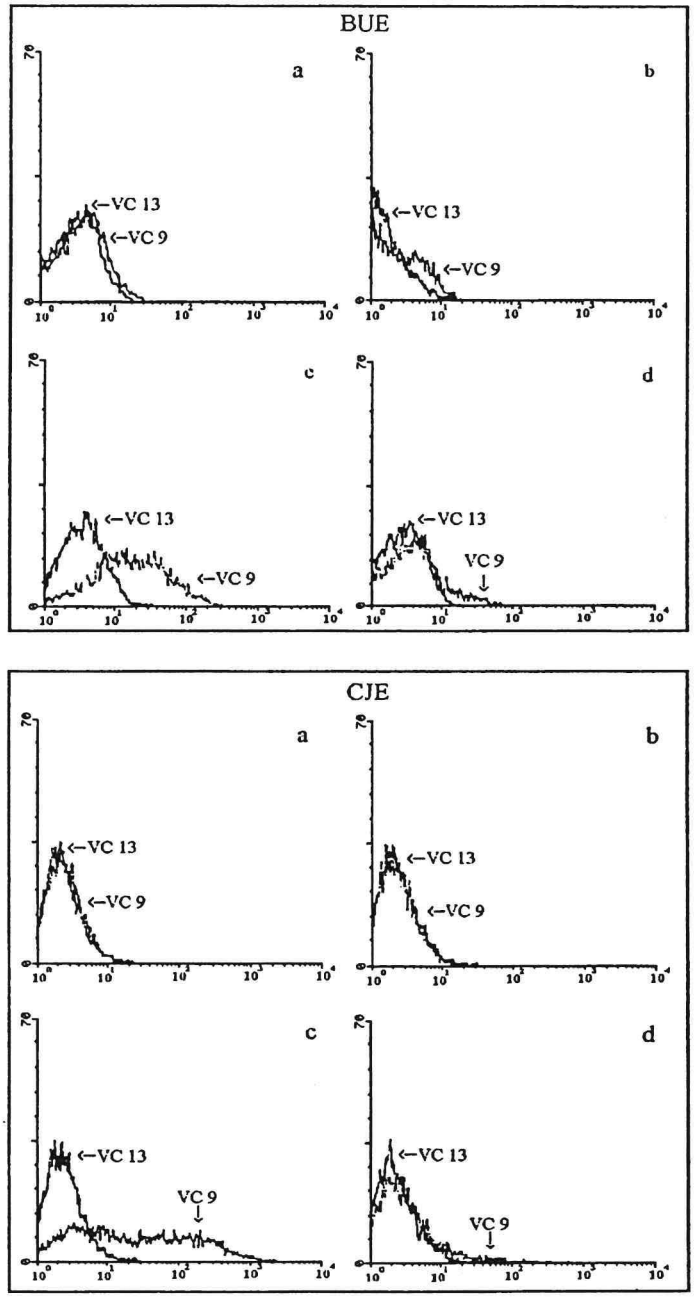

Fig. 5. FACS analysis of BUE and CJE cells. Cells were labelled with an anti-MHC class II mAb (VC9) or a mAb control (VC13) as described in Material and methods. The *Y*-axis represents the number of cells while the *X*-axis corresponds to the intensity of fluorescence; (a) non-infected, (b) *C. ruminantium* infected, (c) non-infected and treated with rBoIFNγ and (d) infected and treated with rBoIFNγ.

ruminantium colonies in endothelial cells. Involvement of oxygen catabolism metabolites, tryptophan degradation, nitric oxide and transferrin receptors in the anti-*cowdria*

effect of rBoIFNγ in vascular endothelial cells is currently under investigation in our laboratory.

The inhibitory effect of IFNγ has been shown to vary between different strains of *R. tsutsugamushi*, with certain strains being unaffected by IFN γ (Hanson, 1991b). Here, we show that three strains of *C. ruminantium* from different parts of the world (Senegal, South Africa and Guadeloupe), with different antigenic properties are each susceptible to rBoIFNγ-mediated inhibition in vitro. This inhibitory effect is independent of the tissue of origin of the endothelial cell lines. Caprine endothelial cells are also equally susceptible to the inhibitory effects of rBoIFNγ.

Thus, rBoIFNγ appears to be a very powerful inhibitor of *C. ruminantium* growth in vitro, acting independently of the rickettsial stock, endothelial cell origin and ruminant species. These results suggest a direct role for IFNγ in protective immune responses against *C. ruminantium* infections.

Endothelial cells have the capacity to process and present antigens to T cells in an MHC class II restricted manner (Sedwick et al., 1990). However, our results suggest that this is not the case during *Cowdria* infections since infected endothelial cells do not express MHC class II molecules and have a markedly reduced capacity to do so even following IFNγ treatment. These results suggest that a direct interaction (leading to cytotoxicity or cytokine production) between $CD4^+$ T cells and *Cowdria*-infected endothelial cells is unlikely in vivo. The involvement of MHC class I restricted CD8 T lymphocytes and CD4 helper cells activated by more conventional pathways (e.g. macrophage) in the immunology and immunopathology of cowdriosis is more likely to occur.

Although endothelial cells infected with *Cowdria* are highly sensitive to treatment with IFNγ resulting in a significant reduction in *Cowdria* growth, treatment fails to induce cell surface MHC class II expression. How a few remaining colonies can influence class II induction by IFNγ is not yet understood. IFNγ may act on the later stage of the *Cowdria* growth cycle while inhibitory factors are produced by earlier stages.

Our results suggest that *C. ruminantium* has developed mechanisms to escape recognition by the host immune response. These include the production of inhibitory factors that greatly reduce the capacity of endothelial cells to express MHC class II molecules. As *C. ruminantium* is extremely sensitive to IFNγ it is likely to have developed means to limit the production of this cytokine. One of these may be to prevent MHC class II restricted activation of Th1 subsets of T lymphocytes that produce IFNγ. Further experiments to study the effect of *Cowdria* infection on MHC class I expression and the constitutive expression of MHC class II molecules by professional antigen-presenting cells (monocytes, dendritic cells) are planned.

Acknowledgements

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Article 3

PROTECTION AGAINST COWDRIA RUMINANTIUM INFECTION IN MICE WITH GAMMA INTERFERON PRODUCED IN ANIMAL CELLS.

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ABSTRACT. We report here that γ interferon produced in animal cell culture injected intraperitoneally, efficiently protects mice against *Cowdria ruminantium* infection. None of the other cytokines tested exerted any effect. Neutralizing antibodies against the cytokines (anti IL6, anti TNF, anti γIFN), did not modify the course of the disease, indicating that these cytokines do not play any crucial role in the pathology. The results concerning the protective effect of interferon pave the way towards the establishment of a rational selection method for protective antigens.

1. Introduction.

1.1. HEARTWATER AND *COWDRIA RUMINANTIUM.*

Heartwater is a tick borne disease of wild and domestic ruminants which is endemic in sub-saharan Africa. The infectious agent causing the disease which is a major obstacle to upgrading of cattle (Uilenberg, 1983), is a rickettsia, a strict obligate intracellular organism replicating in the cytoplasm of endothelial cells of blood capillary vessels which are the final target for the infection.
The disease is generally fatal if not appropriately treated. No really convenient, efficient and safe vaccination procedure is available yet. Indeed, the present procedure requires the use of intraveneous injection of blood from infected animals followed by antibiotherapy at the time symptoms appear. This confers protection to the treated animals for a few months. If Terramycin treatment is made before appearance of the symptoms, no protection develops, meaning that some steps of the infectious cycle should start in order to elicit immunity.

We showed however that α and γ interferons play an important role in the resistance against the disease (Totté, Blankaert et al., 1993 - Totté, De Gee et al., 1993 - Totté, Jongejan et al., 1994).

1.2. THE MURINE MODEL SYSTEM FOR THE RICKETTSIA INFECTION.

A murine model system of the infection with *Cowdria ruminantium* exists. It has revealed that cellular immunity is essential in the mechanism of protection (Du Plessis, 1991).
We used this model also to establish the protective effect of recombinant myeloperoxidase. This indicates that other non specific mechanisms are important in the natural resistance to the rickettsia (Totté et al. 1994).

The model could also be used to establish the potentiality of γ Interferon in the development of an appropriate vaccination procedure. The present study is a first step towards such a goal.

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2. Materials and methods.

2.1. *COWDRIA RUMINANTIUM.*

Welgevonden stock isolated originally from South Africa was obtained from spleer homogenates of mice i.v. infected with 0.1 ml of similar extracts passed from mouse tc mouse and kept frozen in aliquots.

2.3. MICE INFECTION AND TREATMENTS.

NMRI mice of 6 weeks were used for *Cowdria ruminantium* production and for the study of the effect of interferon on the infection.
Infection with the Welgevonden stock of *Cowdria ruminatium* was intravenous (dose from 1 to 6 LD 50 were used, depending of the experiment, as indicated in figure and table).
Interferon from animal cell culture (originating from the Rega Institute production line), was administred intraperitoneally at a dose of 50.000 U / mouse per injection.

3. Results.

γ Interferon produced in animal cell culture was shown to protect efficiently mice against a lethal infection with *Cowdria ruminantium* (Fig.1). A full protection needed daily treatment for the two weeks of the incubation period.Treatment during a more limited time results in only a delay in the onset of the disease.

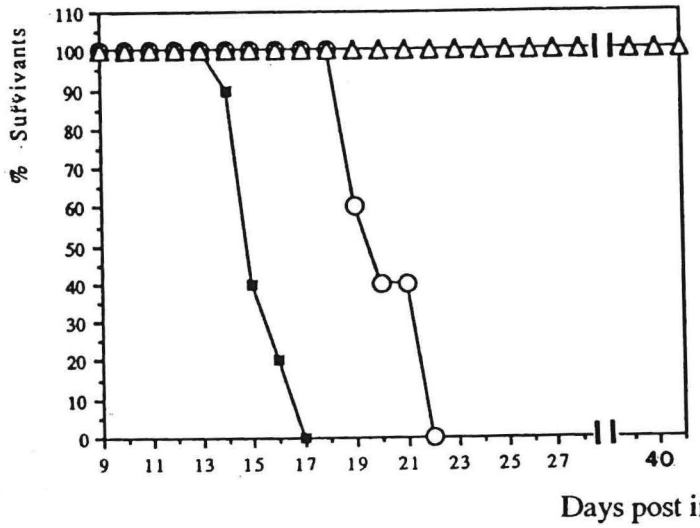

Figure 1. Protection against *Cowdria ruminantium* (2 LD 50) with Gamma interferon (50.000 U / mouse i.p.).
Groups of 10 mice were treated daily at days -1,0,2,4,6,8,10,12,14 and 16 (Δ) or accordig the same schedule, but until day 10 only (O). The control (■) is treated by PBS.

Moreover, the degree of protection depends on the infectious dose (Table 1).
By contrast mouse rec TNF (10.000 U / mouse per day treatment) had no effect on the disease (not shown).

TABLE 1. Effect of γ Interferon against *Cowdria ruminantium* .

Group	Infection and Treatment	Incubation period	Mortality
1	CR Wel 6 LD 50 / IFN (day -1 to 16)	13-14	5 / 5
	CR Wel 6 LD 50 / PBS	13-14	5 / 5
2	CR Wel 2 LD 50 / IFN (day -1 to 16)	_	0 / 5
	CR Wel 2 LD 50 / PBS	14-17	5 / 5
3	CR Wel 2 LD 50 / IFN (day -1 to 10)	19-22	5 / 5
	CR Wel 2 LD 50 / PBS	14-17	5 / 5
4	CR Wel 1 LD 50 / IFN (day -1 to 10)	_	0 / 10
	CR Wel 1 LD 50 / PBS	14-16	6 / 10

Infection is with Welgevonden stock of *Cowdria ruminantium* (CR Wel 1, 2 or 6 LD 50 / NMRI mouse i.v. at day 0). For Groups 1 and 2, IFN treatment (50.000U / mouse /day) were i.p. at days -1,0,2,4,6,8,10,12,14,16. For Groups 3 and 4, IFN treatment was made accordingly, but up to day 10 only.

It should be mentionned (not shown) that none of the cytokine tested in the present sudy (TNF, γ IFN) was detected in the circulation during the infection. Moreover the neutralizing antibodies against TNF, γ IFN and IL6, did not modify the evolution of the disease.

4. Discussion.

We have shown before that in experimental infection in cattle with *Cowdria ruminantium*, partial natural resistance was associated with an early induction of α interferon (Totté, De Gee et al. 1993).No natural resistance of this sort was however observed in mice strains tested up to now. In the bovine system, we showed that α IFN and even more γ IFN were able to protect in vitro endothelial cells against the rickettsia infection (Totté, Blankaert et al. 1993 - Totté, Jongejan et al. 1994).

We showed here that exogenous γ IFN establish in vivo, an antirickettsia state in mice. The treatment should however be sustained for a long period corresponding to the normal incubation time. Surprisingly, knowing the extreme sensitivity of the rickettsia infection observed in vitro in endothelial cells, full protection in this in vivo model, required high dose of interferon. The protective activity of interferon is moreover counteracted when high infectious dose of the rickettsia is used.

TNF has no effect in this system. We showed before that IL6 was induced during the

infectious process. However antibodies against IL6 or against TNF and even γ IFN, do not affect the evolution of the disease, indicating that those cytokines do not play a crucial role in the pathology, in contrast to what was observed in other inflammatory models (Heremans et al. 1989).
We showed in a parallel study (Totté, Moguilevsky et al. 1994) that myeloperoxidase is a very active inhibitor of the development of *Cowdria ruminantium* even when administration is made early after infection. Moreover, the treatment with this recombinant enzyme naturally occuring in azurophilic granules of neutrophils, permitted the establishment of an active immunity to the rickettsia.

The present results indicating that endogenous γ IFN is not playing an important role in a primary infection in NMRI mice, may reflect the development of a possible escape mechanism against interferon by *Cowdria ruminantium* . Such a possibility is suggested by our recent data showing that in bovine endothelial cells, the infection by the rickettsia blocks the expression of MHC class II antigens elicited by γ IFN treatment (Totté, Vachiery et al., unpublished). We have not yet been able after infection in mice , to show in the circulation any increase of IL10, that could explain an anti IFN effect, but a more specific approach should be undertaken in order to evaluate this interesting possibility.
Taken together, our data showing that in our mice model, cytokines are probably more important in secondary *Cowdria infection*, pave the way towards the development of methods that may help in selecting more rationally antigens able to modulate the expression of some important mediators, and by this way to increase their vaccinating potential .

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Article 4

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Analysis of T-Cell Responses in Cattle Immunized against Heartwater by Vaccination with Killed Elementary Bodies of *Cowdria ruminantium*†

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Cattle were successfully immunized against heartwater with a lysate of *Cowdria ruminantium* formulated in Freund's adjuvant. Vaccinated animals proved fully resistant to virulent challenge 3 and 10 months after vaccination. For the first time a helper T lymphocyte response to *Cowdria* antigens was observed and characterized. *Cowdria*-specific T-cell lines generated from vaccinated animals by in vitro restimulation with *Cowdria* lysates are 95 to 100% CD4$^+$, are MHC class II restricted, and produce gamma interferon. They proliferate in response to autologous monocytes infected with live *Cowdria* but not in response to uninfected monocytes. These T-cell lines will facilitate the search for *Cowdria* antigens that are immunogenic for T cells and will therefore be of relevance in the development of a subunit vaccine against the disease.

Cowdria ruminantium is a tick-transmitted rickettsia that causes heartwater, or cowdriosis, an economically important disease of ruminants in sub-Saharan Africa and the Caribbean islands (32).

The available method for vaccination against heartwater consists of administration of live virulent organisms to animals followed by tetracycline treatment during the febrile reaction (4). This infection-and-treatment method is cumbersome and risky, and the search for safer, more practical vaccines against the disease is therefore a priority (32). Attenuation of the pathogen has been reported (11), but the only available attenuated vaccine is based on the Senegalese strain of *C. ruminantium* and does not cross-protect against several other strains (12). More recently, successful immunization with inactivated *Cowdria* organisms emulsified in Freund's adjuvant has been achieved in goats (19) and sheep (16). This suggests that the development of a subunit vaccine against the disease may be feasible. However, identification of defined *Cowdria* antigens for use in a recombinant vaccine will depend on a better understanding of the mechanisms involved in protective immunity and in the pathogenesis of the disease.

Immunological studies of heartwater in ruminants have concentrated solely on antibody responses. In some instances hyperimmune sera from cattle and mice have been shown to neutralize the infection in vitro (6), whereas in others no significant effect was observed (19). Transfer of hyperimmune serum or purified gamma globulins did not confer protection in ruminants (7) or in mice (6). Since *C. ruminantium* is an obligate intracellular pathogen, it is likely that T-cell-mediated immune mechanisms play a role in the protection. Studies with mice have shown that the transfer of Lyt-2$^+$ T lymphocytes from animals immunized by infection and treatment protects susceptible recipients against *Cowdria* infection (8). There are no reports to date on T-cell responses to *Cowdria* infection in ruminants.

In this study, we confirm the feasibility of using killed elementary bodies of *Cowdria* to immunize cattle against heartwater. We have studied cellular aspects of the bovine immune response to *Cowdria* and report on the characterization of a *Cowdria*-specific CD4$^+$ T lymphocyte response in immunized cattle. We also define a system whereby CD4$^+$ T-cell lines can be used to identify *Cowdria* antigens that are immunogenic for T lymphocytes.

MATERIALS AND METHODS

***Cowdria* cultivation and antigen preparation.** *C. ruminantium* (Gardel stock) was grown in bovine umbilical endothelial cells (BUEC) as previously described (18). When the culture showed between 70 and 80% cytopathic effect, the supernatant was collected and cell debris was removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was further centrifuged at 14,000 × *g* for 30 min, and the pellet was resuspended in 0.1 M phosphate-buffered saline, pH 7.1. *Cowdria* elementary bodies were inactivated by five freeze-thaw cycles performed with liquid nitrogen and were stored at −20°C until used for immunization. The resulting preparation was not infective for BUEC, indicating that it did not contain viable organisms (data not shown). For T-cell proliferation assays, *Cowdria* antigens were prepared as above, but after centrifugation at 14,000 × *g* for 30 min the pellet was passed 10 times through a 26-gauge needle and resuspended in sucrose-phosphate-glutamate buffer (5). Organisms were further purified on discontinuous Renografin density gradients (33). After three washes in phosphate-buffered saline, purified organisms were subjected to five freeze-thaw cycles performed with liquid nitrogen and were stored as described above. Lysates of uninfected BUEC were also prepared for control purposes. The protein content of lysates was determined by the Bradford method (Pierce).

Immunization and challenge of animals. Nine 8-month-old Ayrshire cattle were obtained from the ILRI ranch, which practices regular spraying of animals with acaricides. Prebleed sera of all animals were negative for anti-*Cowdria* antibodies as determined by an indirect immunofluorescence assay test (18). Five animals were immunized by intramuscular inoculation of 0.5 ml of killed elementary bodies of *C. ruminantium* formulated in an equal volume of complete Freund's adjuvant (CFA). One month later, the calves received a similar inoculation in incomplete Freund's adjuvant. Each animal received a total of approximately 100 μg of killed *Cowdria*. Two animals were challenged 3 months after boosting (group A) and three were challenged after 10 months (group B) by intravenous injection of 3 ml of a virulent *Cowdria* preparation derived from in vitro cultures. This dose had been shown previously to cause 100% mortality in naive cattle and corresponded to 135 50% tissue culture lethal doses ($TCLD_{50}$) as determined by the 50% culture lytic dose titration method (28). The remaining four animals were used as nonimmunized controls. Previous experiments have shown that neither Freund's adjuvant nor inactivated *Cowdria* alone confers protection in small ruminants against virulent challenge with the agent (16, 20).

Generation of short-term T-cell lines. Peripheral blood mononuclear cells (PBMC) were collected from group B animals 8 months after the primary immunization and 2 months before challenge. Cells were separated by flotation of blood collected in Alsever's solution on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The cells were cultured in 24-well plates, at a density of 10^6/ml, in RPMI medium containing 10% fetal bovine serum, penicillin (100 IU/ml), strep-

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TABLE 1. Outcome of the homologous virulent challenge of vaccinated and naive cattle

Animal	Treatment date (mo/yr)		Days to fever[a]	Maximum temp (°C)	Time to death (days)	Outcome
	Primary immunization	Challenge				
Immunized						
Group A						
322	5/94	8/94	14	40.2	—[b]	Immune
338	5/94	8/94	8	40.7	—	Immune
Group B						
295	5/94	3/95	5	41.0	—	Immune
296	5/94	3/95	5	40.9	—	Immune
304	5/94	3/95	3	41.3	—	Immune
Naive						
109		7/94	13	40.7	16	Death
110		7/94	14	40.3	18	Euthanized[c]
294		8/94	7	41.0	14	Euthanized
264		3/95	8	40.5	12	Death

[a] Number of days between infection and pyrexia (T > 39.5°C).
[b] —, death did not occur.
[c] Euthanized upon appearance of nervous signs.

tomycin (100 μg/ml), L-glutamine (2 mM), 5×10^{-5} M 2-mercaptoethanol (complete medium), and 1 μg of *Cowdria* lysate per ml in a total volume of 2 ml. After culture for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air, viable cells were restimulated with *Cowdria* lysate at a density of 5×10^5 cells/well in complete medium, in the presence of 10^6 autologous gamma-irradiated (500 rads) PBMC as antigen presenting cells (APC). Viable cells were cryopreserved in liquid nitrogen 1 week after the last restimulation. Freshly thawed cell cultures were used in T-cell proliferation assays only if their viability was above 90%.

T-cell proliferation assays. Fresh PBMC were seeded at a density of 3×10^5 cells/well in 96-well flat-bottomed plates in a final volume of 200 μl. For assays with cell lines, wells were seeded with 2×10^4 T cells and 6×10^4 irradiated syngeneic PBMC. Where appropriate, *Cowdria* antigens were included in cultures at a previously determined optimal concentration of 1 μg/ml. On day 4 of the culture period, 0.5 μCi of [^{125}I]iododeoxyuridine (Amersham International, Amersham, United Kingdom) was added to each well and incubated overnight. The cells were harvested onto glass fiber filters with a cell harvester (Flow Laboratories), and the radioactivity was counted with a Beckman 5500 gamma counter. Results are expressed as counts per minute averaged from duplicate wells. In some experiments monocytes infected with live *Cowdria* were used as APC. Monocytes were purified from PBMC by adherence to polystyrene (10), and 3×10^6 cells were infected with the equivalent of 650 $TCLD_{50}$ (28) of freshly prepared elementary bodies of *Cowdria*. The cells were harvested 48 h after infection, fixed in 0.1% glutaraldehyde, and used as APC (10^4 cells/well) in proliferation assays. Electron micrographic studies confirmed that, at the time of harvest, *Cowdria* organisms had differentiated into reticulate bodies within infected monocytes (unpublished data). For blocking experiments, monoclonal antibodies (MAb) specific for bovine class I and class II major histocompatibility complex (MHC) molecules were added to the assay in the form of ascites at a final dilution of 1/500. MAb IL-A88 (immunoglobulin G2a [IgG2a]) recognizes a monomorphic determinant on bovine class I MHC molecules (31), MAb IL-A21 (IgG2a) is specific for class II MHC DR (9), and MAb VC9 recognizes both class II MHC DQ and DR subtypes (3, 30).

FACS analysis. The surface phenotype of PBMC and T-cell lines was analyzed by indirect immunofluorescence staining using specific MAb. MAb IL-A12 (1), IL-A51 (14), and GB21A (15) recognize bovine CD4, CD8, and γδ T-cell receptor, respectively. MAb IL-A30 (34) recognizes bovine cell surface IgM and therefore defines B cells. MAb IL-A111 recognizes the bovine interleukin 2 (IL-2) receptor (22). MAb J11 (2) recognizes a monomorphic determinant on bovine class II MHC molecules. Cells were stained as described by MacHugh et al. (14) and analyzed on a fluorescence-activated cell sorter (FACS) (FACS II, Becton Dickinson, Sunnyvale, Calif.). Results are expressed as the percentage of total cells above background level that fluoresced.

Bovine IFN-γ assay. Bovine gamma interferon (IFN-γ) in culture supernatants was detected with a specific enzyme-linked immunosorbent assay kit (CSL, Parkville, Australia) and quantified against recombinant bovine IFN-γ (CIBA-GEIGY). Assays were performed on day 4 supernatants, which were observed in pilot experiments to reflect maximal expression.

RESULTS

Clinical responses of cattle to *Cowdria* challenge. All immunized animals survived virulent challenge (Table 1), showing no clinical signs other than transient fever. In contrast, all naive challenged control animals developed heartwater and either died without overt clinical signs (peracute heartwater) or were euthanized upon appearance of nervous signs ranging from incoordination to lateral recumbency with opisthotonus (acute heartwater). Brain smears prepared from all naive animals at autopsy were positive for colonies of *Cowdria*. No significant differences were observed between immunized and naive animals in the duration and magnitude of the febrile reaction.

Characterization of the proliferative response to *Cowdria* Ag. When tested prior to challenge, PBMC from all immunized animals responded strongly to *Cowdria* antigens (Ag). Proliferative responses of PBMC taken during the course of immunization from animals 338 and 295, which are representative individuals from groups A and B, respectively, are illustrated in Fig. 1. No proliferation was observed prior to immunization or in naive controls. PBMC from *Cowdria*-immunized animals responded to uninfected BUEC Ag, but the proliferation was consistently lower than the response observed with *Cowdria* Ag. This was attributed to the fact that the animals were immunized with partially purified *Cowdria* Ag, which undoubtedly also contained BUEC Ag. Boosting the inoculation did not significantly increase the level of the response to *Cowdria* Ag.

The phenotype of responding lymphocyte populations was determined by FACS analysis after 5 days of incubation with the different Ag preparations. Incubation of PBMC with *Cowdria* Ag resulted in an increase in relative cell numbers in only the $CD4^+$ fraction (Table 2). The proportion of lymphoblasts also dramatically increased in the $CD4^+$ fraction as well as in other populations but to a lesser extent, and for the latter without any increase in relative numbers. In contrast, when PBMC were incubated with BUEC Ag, the majority of responding cells were γδ T lymphocytes (data not shown). The expression of class II MHC molecules and IL-2 receptors on the surfaces of T cells responding to the different stimuli was also examined (Table 2). An increase in the expression of both class II MHC and the IL-2 receptor was observed only in the $CD4^+$ fraction and only after incubation with *Cowdria* Ag, which is consistent with an active proliferative response. Other lymphocyte populations in the culture also expressed higher levels of class II MHC molecules but did not up-regulate IL-2

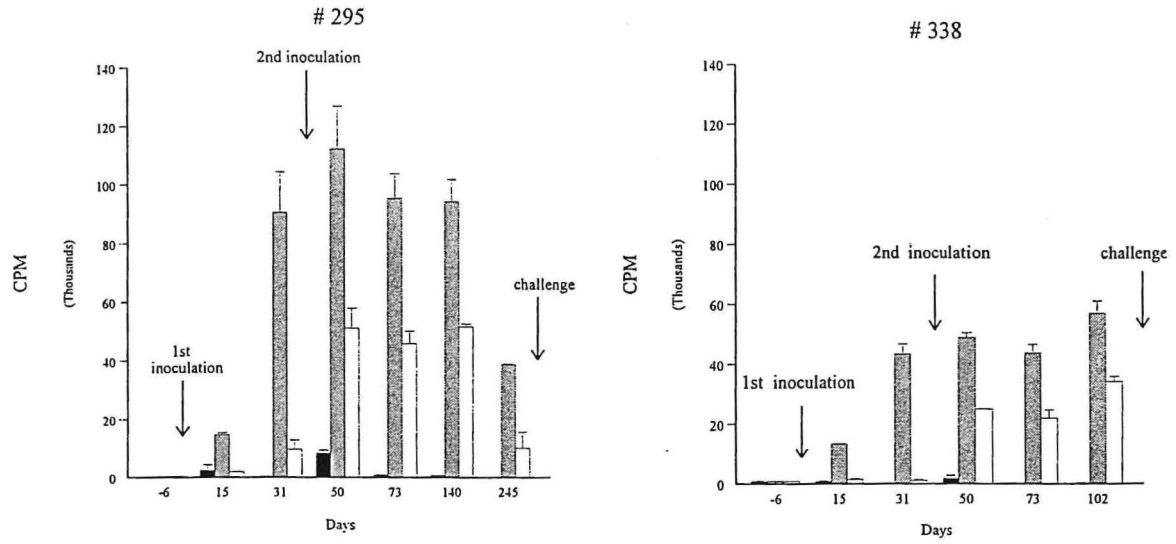

FIG. 1. Proliferative response of PBMC from two *Cowdria*-immunized cattle (295 and 338). PBMC were incubated with medium (black bars), inactivated *Cowdria* (grey bars), and uninfected BUEC lysate (white bars). Both *Cowdria* and BUEC Ag were used at a final concentration of 1 μg/ml. The results represent means plus standard deviations of duplicate cultures.

receptor expression. This is in line with the observation that a proportion of $CD8^+$ and γδ T cells were enlarged but did not proliferate in response to *Cowdria* Ag. Increased expression of class II MHC and the IL-2 receptor after stimulation of PBMC with BUEC Ag was observed in γδ T cells only (data not shown). Although FACS analysis revealed an unexpectedly high proportion of sorted $CD4^+$ T cells expressing class II MHC molecules after culture in medium alone (Table 2), the level of fluorescence was low in comparison to that for cells incubated with *Cowdria* Ag.

Generation and characterization of *Cowdria*-specific T-cell lines. After three restimulations at weekly intervals with *Cowdria* lysate, cell lines proliferated in response to *Cowdria* Ag but not to BUEC Ag (Table 3). They also recognized autologous monocytes infected with the live pathogen (Table 3). When analyzed 1 week after the third restimulation, these *Cowdria*-specific short-term cell lines were 95 to 100% $CD4^+$ T lymphocytes, as shown by FACS analysis, with the remaining cells being $CD8^+$ (Fig. 2). Maximal proliferation occurred at day 4 and required the presence of autologous irradiated PBMC as APC. No proliferation was observed when heterologous PBMC were used as APC, suggesting that the response was not the result of a mitogenic factor (data not shown). Blocking experiments using MAb confirmed that proliferation was MHC class II restricted (Table 4). A combination of two anti-MHC class II antibodies was necessary to completely abrogate the proliferation, suggesting that both the DQ and DR subtypes of MHC class II are involved.

TABLE 2. T-cell phenotype and Class II MHC and IL-2 receptor surface expression after 5 days of culture with medium and *Cowdria* antigens

Animal no.	Days after boosting	Cell subpopulations (% total PBMC)[a]						CII[b,c] and IL-2R[d] expression (% positive cells)											
		CD8		CD4		γδ		CD8				CD4				γδ			
								M		CAg		M		CAg		M		CAg	
		M[e]	CAg[f]	M	CAg	M	CAg	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R
295	140	12	10	28	42	22	20	13	8	23	4	20	13	90	31	8	8	28	10
		26	**45**	**20**	**78**	**23**	—[g]												
296	140	18	18	30	44	24	28	20	6	35	5	17	6	64	25	12	6	39	6
		20	**57**	**10**	**73**	**12**	**34**												
304	140	20	14	25	38	26	23	26	3	23	1	23	10	80	38	29	7	38	8
		40	**46**	**20**	**76**	**22**	**56**												
322	33	22	15	22	38	16	17	—	—	—	—	30	15	83	50	—	—	—	—
		30	**71**	**33**	**84**	**50**	**76**												
338	33	16	17	20	39	16	20	—	—	—	—	15	8	52	29	—	—	—	—
		25	**66**	**22**	**82**	**22**	**52**												

[a] Boldface numbers, % lymphoblasts of a given phenotype.
[b] CII, class II MHC.
[c] Class II MHC-positive cells in control cultures were expressed at low levels.
[d] IL-2R, IL-2 receptor.
[e] M, medium.
[f] CAg, *Cowdria* Ag.
[g] —, not done.

TABLE 3. Specificity of *Cowdria*-responsive T-cell lines

Cell line and no. of restimulations	Proliferative response (cpm)[a]				
	Medium	*Cowdria* lysate	BUEC	Monocytes −[b]	Monocytes +[c]
295					
0	555 ± 66	38,764 ± 177	10,080 ± 5,448	—[d]	—
3	1,080 ± 102	53,584 ± 1,869	601 ± 30	1,198 ± 282	28,438 ± 2,394
296					
0	657 ± 26	26,665 ± 4,924	6,401 ± 1,158	—	—
3	1,290 ± 880	19,836 ± 1,628	949 ± 343	1,210 ± 97	17,715 ± 713
304					
0	213 ± 118	20,623 ± 3,279	6,925 ± 1,485	—	—
3	965 ± 262	40,689 ± 3,742	896 ± 100	—	—

[a] Results are means ± standard deviations of duplicate cultures.
[b] Monocytes −, uninfected monocytes.
[c] Monocytes +, monocytes infected with live *Cowdria*.
[d] —, not done.

Upon stimulation with *Cowdria* Ag, these T-cell lines secreted significant levels of IFN-γ into the medium (Table 5). Although we cannot exclude the possibility that contaminating $CD8^+$ T cells also produced IFN-γ in these cultures, cell line 296, which had the highest percentage of $CD8^+$ T cells, was the lowest IFN-γ producer.

DISCUSSION

We have confirmed the feasibility of immunizing cattle against heartwater by using killed *Cowdria* organisms. Five cattle were solidly protected against homologous challenge, and three survived a lethal challenge 10 months after immunization. Immunized animals showed no signs of illness after challenge other than an early febrile reaction. This method of vaccination has also protected goats (19) and sheep (16) and is a significant improvement on the infection-and-treatment technique used to date. No tetracycline treatment is required, and all inoculations are intramuscular, abrogating the requirement for trained veterinarians to perform the immunizations. In addition, because the method is based on killed *Cowdria* organisms, it can be used in areas where the disease has spread but is not yet endemic. Finally, because the vaccine is produced in vitro in endothelial cell cultures, the risk of it transmitting other diseases is considerably reduced. Efforts are currently focused on the transfer of this method from the laboratory to the field. This will undoubtedly be hastened by the observation that CFA, which is unacceptable for use in food animals, can be replaced with an acceptable, commercially available adjuvant without loss of protection in goats (20). Success of this immunization method in the field will, however, depend on its capacity to protect against heterologous challenge or on whether a cocktail of antigenically different strains of *Cowdria* can provide comprehensive protection. It will also be necessary to determine the optimal dose of killed *Cowdria* organisms

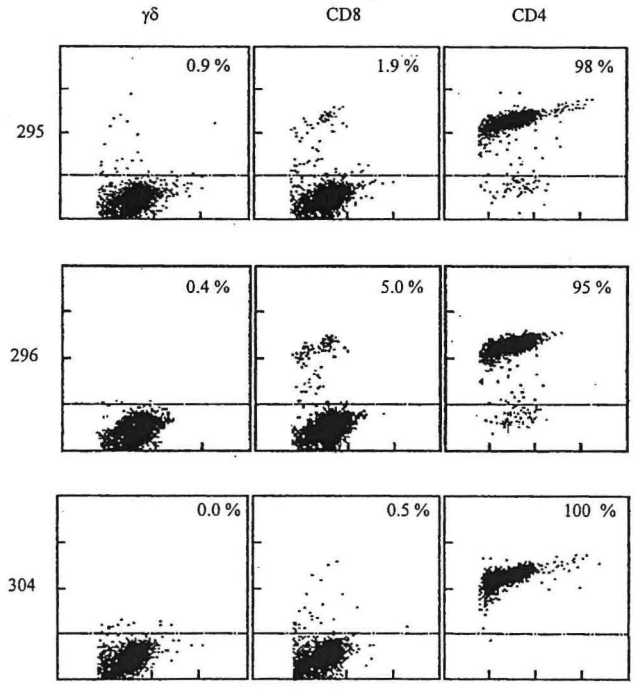

FIG. 2. Phenotypes of bovine *Cowdria*-specific T-cell lines. FACS analysis was performed with specific MAb against bovine CD4, CD8, and γδ T-cell receptor. The *y* axis represents the intensity of fluorescence (log scale) and the *x* axis represents cell size (forward scatter, linear scale). Results are expressed as the percentage of total cells above background level (indicated by the horizontal line) that fluoresced.

TABLE 4. Effect of MHC-specific MAb on proliferation of bovine *Cowdria*-specific T-cell lines

Cell line	Specificity of blocking MAb	T-cell response (cpm)[a]	
		Cowdria Ag −[b]	*Cowdria* Ag +[c]
295	Medium	4,100 ± 512	64,224 ± 625
	Class I	—[d]	67,445 ± 1,025
	Class II DQ+DR (1)	—	33,519 ± 2,153
	Class II DR pan (2)	—	28,906 ± 962
	(1) + (2)[e]	—	8,333 ± 176
296	Medium	2,860 ± 900	23,598 ± 1,333
	Class I	—	24,192 ± 2,887
	Class II DQ+DR (1)	—	15,040 ± 705
	Class II DR pan (2)	—	10,577 ± 911
	(1) + (2)	—	4,032 ± 446

[a] Results are means ± standard deviations of duplicate experiments.
[b] *Cowdria* Ag −, in the absence of *Cowdria* Ag.
[c] *Cowdria* Ag +, in the presence of *Cowdria* Ag.
[d] —, not done.
[e] (1) + (2), class II DQ + DR and class II DR pan.

TABLE 5. IFN-γ production by *Cowdria*-specific T-cell lines[a]

Cell line	IFN-γ production (ng/ml)[b]	
	−	+
295	<0.02	12.5 ± 2
296	<0.02	2.5 ± 1
304	<0.02	13.8 ± 3

[a] T-cell lines were cultured for 4 days in the presence (+) or absence (−) of inactivated *Cowdria*.
[b] Values are means ± (where appropriate) standard deviations.

required for protection against tick-delivered challenge. Although it is likely that the dose of inactivated *Cowdria* can be reduced, large quantities of endothelial cells will still be needed to produce it. This might be achieved through the culture of endothelial cells on collagen microspheres in bioreactors (26). In the longer term, however, the use of recombinant *Cowdria* proteins produced by transformed bacteria is a more practical solution. Reagents generated in the course of this study provide a solid basis for the identification of immunoprotective Ag of *Cowdria*. The available information on immunity to heartwater and to intracellular pathogens in general would suggest that cellular immune responses play an important role in protection. Previous immunological studies on heartwater in ruminants have focused entirely on antibody responses; we have now investigated the capacity of cell-mediated responses to protect against the agent.

We observed that PBMC from vaccinated animals proliferated vigorously in response to *Cowdria* Ag in vitro. No proliferation was observed before vaccination or in naive controls. *Cowdria* Ag preferentially stimulated immune $CD4^+$ T lymphocytes to proliferate. Stimulation of $CD4^+$ T cells was dependent on irradiated APC, and exogenous growth factors were not required. Responding cells expressed high levels of class II MHC molecules and the IL-2 receptor on their surfaces. Repeated stimulation of PBMC with *Cowdria* lysates allowed enrichment of this cell type in short-term T-cell lines. Unlike freshly prepared immune PBMC, these lines did not respond to uninfected BUEC Ag and were therefore entirely specific for *Cowdria* Ag. They also proliferated in response to autologous monocytes infected with live *Cowdria* organisms. This observation may have significance for the activation of these responses during challenge. Although there is no evidence that *Cowdria* replicates within monocytes or macrophages in vivo, extracellular *Cowdria* organisms are found in the blood of infected animals (23) and, therefore, are available for phagocytosis by monocytes and presentation to the immune system.

Induction of a $CD4^+$ T-cell response to *Cowdria* Ag by immunization with killed *Cowdria* formulated in CFA was predictable since inactivated or lysed organisms are likely to be processed for presentation in association with MHC class II molecules. Nonetheless, class I MHC-restricted $CD8^+$ T-cell responses to immunization with exogenous, nonreplicating agents and soluble antigens have been reported (25, 35). The possibility that this is the case for animals immunized with inactivated *Cowdria* is currently being investigated.

Although the mechanisms that conferred protection in these animals have not yet been determined, our results are consistent with the involvement of $CD4^+$ T lymphocytes. This population could affect *Cowdria* infection at different levels. The maturation of antibody responses is known to require the input of helper T lymphocytes. It has been shown previously that animals immunized with inactivated *Cowdria* in CFA have higher antibody titers than those vaccinated with inactivated organisms only (16), although a separate study revealed that antibody levels of protected and nonprotected vaccinated animals were similar (19). Whether the quality rather than the quantity of antibodies produced is important for protection has yet to be investigated. $CD4^+$ T cells may also provide help for the generation of cytotoxic $CD8^+$ T lymphocytes by secreting stimulatory factors and/or through cell contact (13, 21). This observation has recently been confirmed for cattle infected with the intracellular protozoan *Theileria parva* (24). Finally, $CD4^+$ T lymphocytes could also contribute to protection through the release of cytokines such as IFN-γ. We have reported previously that recombinant bovine IFN-γ is a potent inhibitor of *Cowdria* growth both in vitro (27, 30) and in vivo (29). Endogenous IFN-γ produced by concanavalin A-stimulated PBMC was also found to inhibit the growth of *Cowdria* in vitro (17). We have now established that *Cowdria* Ag can specifically induce $CD4^+$ T lymphocytes to produce IFN-γ. The availability of *Cowdria*-specific $CD4^+$ T-cell lines will now enable us to identify the components of the agent that provokes these responses. These Ag would have clear potential for the development of a subunit vaccine against the disease.

In summary, we have shown that PBMC from animals rendered resistant to *Cowdria* challenge by vaccination with inactivated organisms contain *Cowdria*-specific, MHC class II-restricted, IFN-γ-producing, $CD4^+$ T lymphocytes. These cells proliferate in response to autologous monocytes infected in vitro with live *Cowdria*. It remains to be shown whether this population also responds in vivo to live *Cowdria* challenge. This is currently being addressed through the use of lymphatic cannulation techniques in immune animals under challenge.

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Bovine CD4$^+$ T-cell lines reactive with soluble and membrane antigens of *Cowdria ruminantium*

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Abstract

Cowdria-specific CD4$^+$ T-cell lines generated from immunised cattle respond to both soluble and membrane proteins of the agent. Furthermore, the lines produced the *Cowdria*-inhibitory cytokine IFN-γ in response to soluble antigens fractionated by gel filtration and FPLC. Activity eluted as a single peak around fraction 15 for all T-cell lines tested. This fraction induced the highest production of IFN-γ by the lines and was shown by SDS-polyacrylamide gel electrophoresis and silver staining analysis to contain less than 10 different bands ranging from 22 to 32 kDa. Given their high sensitivity and specificity, these short-term CD4$^+$ T-cell lines will be valuable tools for the identification of *Cowdria* antigens for incorporation in a subunit vaccine.

Keywords: *Cowdria ruminantium*; CD4$^+$ T cell lines; IFN-γ; Immunogenic proteins; Fast performance liquid chromatography (FPLC)

1. Introduction

Cowdria ruminantium is a tick-transmitted intracellular bacterium that causes the disease cowdriosis, or heartwater, which is a major constraint to the improvement of livestock productivity in sub-Saharan Africa and the Caribbean islands (Camus et al., 1996). The available method of immunisation, which relies on infection with live organisms followed by treatment, has several major drawbacks and the development of

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improved vaccines is, therefore, a priority (Uilenberg, 1983). We have adopted a strategy of identifying immunoprotective proteins of the agent for incorporation in a recombinant sub-unit vaccine. To date, immunoscreening tools for this purpose have been limited to immune sera and monoclonal antibodies. However, previous studies have indicated that specific antibodies are insufficient to provide protection against the pathogen (Byrom et al., 1993; Du Plessis, 1970). This has led to the belief that cell-mediated immune mechanisms play an important role in protection against cowdriosis. There is, therefore. a requirement for cellular screening tools for the identification of potentially immunoprotective antigens of the agent.

Observations that immunisation of goats (Martinez et al., 1994) and sheep (Mahan et al., 1995) with inactivated *Cowdria* formulated in Freund's adjuvant confers protection against challenge suggest that helper T-lymphocytes are involved in immunity. We have shown previously that *Cowdria*-specific T-cell lines derived from cattle immunised in this way are 95–100% $CD4^+$, are MHC class II-restricted, and produce IFN-γ (Totté et al., 1997). Although a role for $CD4^+$ T-lymphocytes in protection against *Cowdria* remains to be established in vivo, the inhibitory effect of IFN-γ on the growth of *Cowdria* is well documented (Totté et al., 1994, 1996; Mahan et al., 1996) and provides a rationale for targeting this population in novel vaccine strategies against the disease. In this study, we show that the specificities recognised by bovine $CD4^+$ T-cell lines derived from animals vaccinated with killed organisms and raised against lysates of *Cowdria* are present in both soluble and membrane fractions of the agent. In addition, we have assessed the suitability of these lines to serve as cellular tools for the identification of IFN-γ inducing proteins of the organism.

2. Materials and methods

2.1. Cowdria antigen preparations

A total lysate of *Cowdria ruminantium* (Gardel stock) was prepared as described previously (Totté et al., 1997). Briefly, bovine umbilical endothelial cells (BUEC) were infected with the Gardel stock of the agent and allowed to proceed to 70–100% lysis. The supernatant was collected and cleared of cell debris by centrifugation at 1000*g* for 10 min. After a further centrifugation at 14 000*g* for 30 min, the pellet containing elementary bodies (EBs) of *Cowdria* was collected and resuspended in sucrose–phosphate–glutamate buffer. EBs were further purified on discontinuous Renografin (Sigma) density gradients before being resuspended in 0.1M phosphate buffered saline (PBS), lysed by five freeze–thaw cycles in liquid nitrogen and stored at −20°C. Identical lysates were prepared from uninfected BUEC for control purposes. For the preparation of high–speed supernatant (HSS) and membrane fractions (M), the 14 000*g* EB pellet was resuspended in PBS containing 25 μg/ml of the protease inhibitors Leupeptin and E64 (Cambridge Research Biochemicals, UK). The preparation was sonicated using a Sonifier cell disrupter and ultracentrifuged at 14 000*g* for 2 h to pellet insoluble membrane proteins (M fraction). Soluble proteins were retained in the HSS fraction. HSS

and M fractions were similarly prepared from uninfected BUEC for use as control antigens.

2.2. Animals and generation of $CD4^+$ T-cell lines

Five 8-month-old Ayrshire cattle were used in this study. Three animals were immunised by intramuscular inoculation of killed *Cowdria* in complete Freund's adjuvant, followed, one one month later, by a second inoculation in incomplete Freund's adjuvant. All three vaccinated animals proved fully resistant to virulent challenge whereas naive controls developed heartwater and died, or were euthanised. PBMC were collected two months (Animals 5 and 15) and eight months (Animal 295) after the primary immunisation and two months before challenge. PBMC were restimulated three times in vitro at weekly intervals with 1 μg/ml EB lysate, as described previously (Totté et al., 1997). Briefly, after each stimulation viable cells were restimulated with *Cowdria* lysate in the presence of autologous gamma-irradiated (3000 rads) PBMC as APC. Viable cells were cryopreserved in liquid nitrogen one week after final restimulation. All T-cell lines generated were more than 90% $CD4^+$, as shown by flow cytometry analysis using lineage-specific monoclonal antibodies.

2.3. Fractionation of Cowdria proteins

Soluble *Cowdria* proteins (HSS) were further fractionated by FPLC using a Superose 12 column (Pharmacia). A total of 250 μg of proteins in 300 μl PBS was applied to the column and eluted with PBS at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected, sterilised by UV irradiation, aliquoted, and stored at −80°C before being tested in lymphoproliferation assays. A 50 μl aliquot of selected fraction was analysed by SDS-PAGE using a 10–15% polyacrylamide gradient gel.

2.4. Lymphoproliferation assays

Cell lines were seeded in duplicates at a density of 2×10^4 cells per well along with 6×10^4 irradiated autologous PBMC in round-bottomed 96-well plates. Antigen preparations were included in cultures at a previously determined optimal concentration of 1 μg/ml. FPLC fractions were added to a final dilution of 1 : 4. Proliferation was estimated after four days of culture by measuring IUdR uptake. Results are expressed as counts per minute (CPM), or as stimulation indices calculated as the ratio of CPM measured in test wells to those containing no antigen.

2.5. Bovine IFN-γ assay

Bovine IFN-γ in culture supernatants was detected using a commercial ELISA kit (Commonwealth Serum Laboratories, Parkville, Australia) and quantified against a recombinant bovine IFN-γ (Ciba-Geigy).

3. Results

3.1. Proliferative response of CD4+T-cell lines to Cowdria antigens

All CD4+ T-cell lines generated against *Cowdria* lysate proliferated vigorously in response to the different preparations of *Cowdria* antigen, but not to medium nor uninfected BUEC lysate (Table 1). Both soluble (HSS) and non-soluble (M) preparations of the agent induced a significant proliferation of the lines. No proliferation was observed when heterologous-irradiated PBMC were used as APC, excluding any mitogenic factor. The proliferation of cell line no. 295 was shown previously to be restricted by MHC class II molecules (Totté et al., 1997).

3.2. Stimulatory activity of FPLC-derived fractions of Cowdria

Two different HSS preparations of *Cowdria* were fractionated by gel filtration using FPLC. The fractions obtained in the first experiment were screened using cell line no. 5, whereas fractions obtained in the second fractionation were screened using cell lines nos. 15 and 295. Similar results were obtained in both cases (Fig. 1). Greatest amounts of HSS proteins resolved to fraction 14 (Fig. 1, thin line) with little activity detected before fraction 13 indicating a majority of small proteins around 30 kDa or less according to the standard calibration curve of the column (not shown). Proliferation of all three *Cowdria*-specific CD4+ T-cell lines to the fractions was observed as a single peak, with greatest activity occurring in fraction 15 (Fig. 1). Titration of the fractions did not modify the unimodal aspect of the proliferation curve (i.e., with a peak around fraction 15). No proliferation occurred when heterologous PBMC were used as APC, indicating that non-specific mitogens are not involved. In addition, corresponding FPLC fractions obtained from uninfected BUEC contained no activity (data not shown).

3.3. Induction of IFN-γ by FPLC-derived fractions

Proliferative activity of the cell lines to the active (HSS) fractions was reflected by the IFN-γ titers in supernatants collected from the test cultures (Fig. 2). The concentrations of

Table 1
Specificity of *Cowdria*-responsive CD4+ T-cell lines

Cell line no.	Proliferative response (mean CPM +SD) after incubation with the following antigens				
	Medium	BUEC[a]	*Cowdria* lysate	HSS[b]	M[c]
5	1 700 + 205	1 235 + 11	26 302 + 3 180	8 246 + 1 318	21 914 + 3 068
5H[d]	2 000 + 145	1 880 + 127	2 102 + 355	1 500 + 91	1 972 + 36
15	3 571 + 781	3 592 + 70	98 798 + 2 293	37 837 + 8 495	66 450 + 2 375
15H[d]	2 594 + 60	2 410 + 273	3 015 + 500	1 999 + 311	2 875 + 3 21
295	1 080 + 102	601 + 30	53 584 + 1 869	6 845 + 492	23 648 + 1 340

[a] BUEC: lysate of uninfected endothelial cells.
[b] HSS: high speed supernatant (soluble *Cowdria* proteins)
[c] M: membrane fraction (non-soluble *Cowdria* proteins).
[d] H: irradiated heterologous PBMC were used as APC.

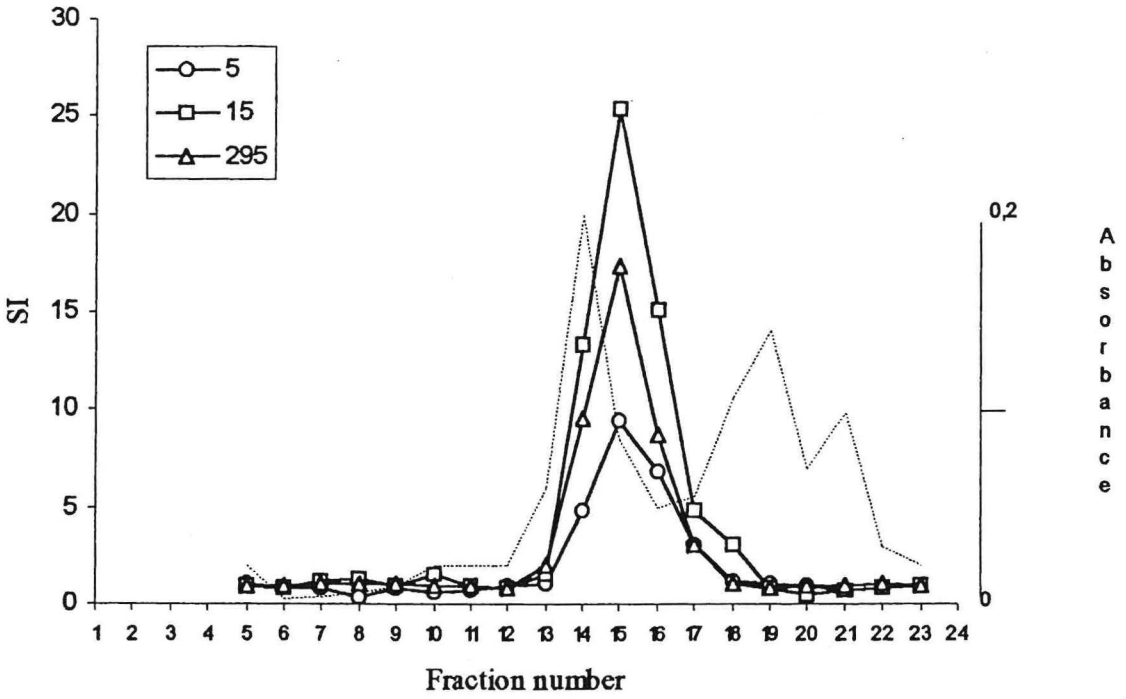

Fig. 1. Proliferative responses of bovine immune CD4$^+$ T-cell lines to soluble *Cowdria* proteins fractionated by gel filtration using FPLC. A total of 250 μg of protein was applied onto a Superose 12 column, eluted with PBS, and collected in 1 ml fractions. Protein content was monitored as absorbance at 280 nm on a scale of 0–0.2 units (thin line). Proliferation of three individual lines to 50 μl of each fraction is illustrated in the form of stimulation indices.

IFN-γ induced by fractions 14–17 were similar, or higher, than those shown previously to inhibit the growth of *Cowdria* in vitro (Totté et al., 1996).

3.4. Analysis of FPLC-derived fractions by electrophoresis

SDS-PAGE and silver staining analysis of fractions selected from the second experiment revealed the presence of protein bands ranging from 13 to 70 kDa. As shown in Fig. 3, those fractions that induced proliferation and IFN-γ production by the cell lines contained bands ranging from 22 to 32 kDa. As expected from Fig. 1, fraction 14 contained the highest amount of proteins. Proteins eluting around fractions 19 correspond to an estimated size of 10 kDa and, therefore, were not visible on the gel. There was a reduction in the number of bands from fractions 14 to 16, indicating that partial purification of stimulatory proteins was achieved. Fraction 16 contained relatively little stained proteins, but stimulated proliferation and IFN-γ secretion quite well (Figs. 1 and 2), thus underlining the high sensitivity of the lines. The major bands at 32 and 22 kDa are likely to be the major antigenic protein 1 (MAP1) and MAP2 of *Cowdria* that have been described previously (Jongejan and Thielemans, 1989; Mahan et al., 1994).

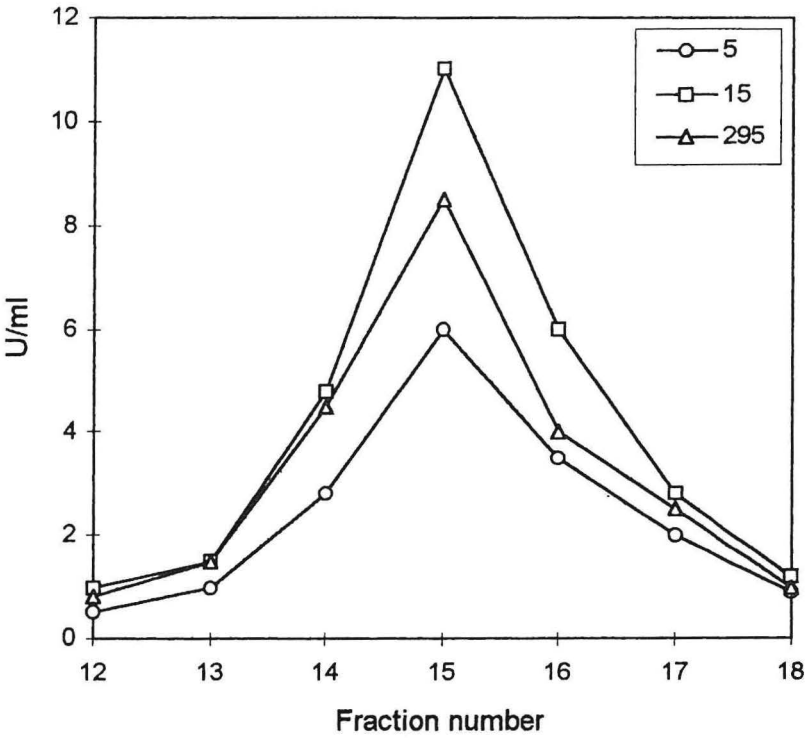

Fig. 2. IFN-γ production by bovine *Cowdria*-specific T-cell lines cultured for 4 days in the presence of selected FPLC fractions.

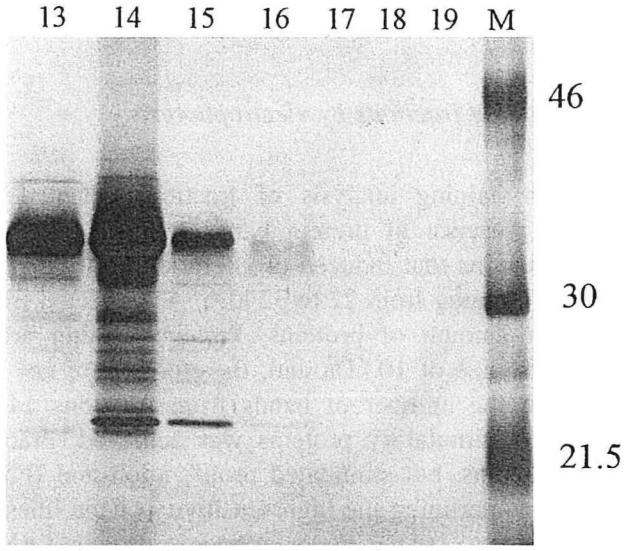

Fig. 3. SDS-PAGE and silver staining analysis of selected FPLC fractions of soluble *Cowdria* proteins. Fraction numbers are indicated above each lane. (M: molecular size markers, in kDa).

4. Discussion

Cellular tools are needed to help identify immunogenic proteins of *Cowdria* for incorporation in a subunit vaccine. Among these, IFN-γ producing $CD4^+$ T-lymphocytes are of considerable interest because of the very potent inhibitory effect of this cytokine on the growth of *Cowdria*. In a previous study, we reported that in cattle immunised against *Cowdria* by vaccination with killed organisms, $CD4^+$ T-cells are capable of producing IFN-γ in response to lysates of the agent (Totté et al., 1997). The data presented in the present study indicate that the specificities recognised by bovine $CD4^+$ T-cell lines raised against *Cowdria* lysates are present in both soluble and membrane fractions of the agent. In addition, we show here that the sensitivity of the lines is adequate for the detection of these determinants in very low concentrations.

Using gel filtration and FPLC, we have determined that the soluble antigen(s) recognised by all three cell lines reside(s) in the 22–32 kDa molecular size range, and confirm that they give rise to the production of IFN-γ by responding cells. However, precise definition of the antigenic specificity of the lines will require further resolution of the activity within the lysate. For example, by using a gel filtration matrix of a pore size more appropriate to the molecular range in question, possibly followed by ion exchange chromatography of active fractions with salt or pH gradients. Nevertheless, we can reasonably exclude MAP1 as a target antigen for these T-cell lines since the 32 kDa band is the dominant protein in fraction 13, which neither induced proliferation nor IFN-γ production. This is in agreement with the observation we made that T-cell lines nos. 5 and 15 do not recognise recombinant MAP1 and that, in animals immunised with killed *Cowdria*, proteins other than the serologically immunodominant MAP1 and MAP2 are strongly immunogenic for $CD4^+$ T-lymphocytes (Totté et al., 1998). In contrast, Mwangi et al. (1998) were able to derive MAP1-specific $CD4^+$ T-cell lines from cattle immunised with live *Cowdria*. In addition, partial protection of mice has recently been achieved by DNA immunisation with MAP1 (Nyika et al., 1998). However, it is unlikely that MAP1 alone will provide sufficient protection against virulent challenge. Thus, cellular reagents such as those developed in this study will facilitate the search for other potentially protective proteins and related genes.

No activity could be detected in FPLC fractions of membrane proteins of *Cowdria* solubilized in SDS. This is likely to be due to protein loss after removal of SDS with extracti-gelcolumns (Pierce). Attempts were made at fractionating membrane proteins of *Cowdria* by SDS-PAGE, followed by transfer onto nitrocellulose. However, nitrocellulose flocules never induced any measurable proliferation of the lines. Continuous-flow electrophoresis is another method that has successfully been used for *Babesia bovis* (Brown et al., 1995) and that is currently being tested for *Cowdria ruminantium* (Van Kleef, personal communication).

In conclusion, this study indicates that immune $CD4^+$ T-cell lines raised against *Cowdria* lysates are highly specific and sensitive. They will be valuable tools for the identification of IFN-γ inducing antigens of *C. ruminantium*. Additionally, we have shown that gel filtration combined with FPLC allows fractionation of soluble proteins of *Cowdria* while retaining their immunogenicity.

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Immune Responses to *Cowdria ruminantium* Infections

P. Totté, A. Bensaid, S.M. Mahan, D. Martinez and D.J. McKeever

Understanding the basis of protective immunity to Cowdria ruminantium *will facilitate the development of an effective subunit vaccine against heartwater in ruminants and contribute to a better definition of protective immune mechanisms to obligate intracellular pathogens in general. Until recently, immunological studies of heartwater in ruminants concentrated solely on antibody responses. Since 1995, the mechanisms underlying cell-mediated immunity of heartwater have been analysed. Progress achieved in these areas is discussed here by Philippe Totté and colleagues, with special emphasis on ruminants, the natural hosts of* C. ruminantium.

Cowdria ruminantium is a tick-transmitted obligate intracellular rickettsia (see Poster, this issue) that causes the economically important disease heartwater, or cowdriosis, in wild and domestic ruminants of sub-Saharan Africa and some of the Caribbean islands[1]. *Cowdria ruminantium* preferentially invades vascular endothelial cells (ECs) and is taken up by neutrophils and monocytes. However, there is no evidence that this pathogen actually replicates within granulocytes in the ruminant host. The pathology of the disease differs between breeds of ruminants and *Cowdria* isolates but is thought to be mainly the result of increased capillary permeability leading to transudation and oedema[2]. Very little direct lysis or cytopathic changes are observed in infected endothelial cells of moribund animals and growing evidence suggests that immune effectors, such as cytokines, play a role in the pathology (see below). Animals that recover from the infection develop a strong and long-lasting immunity to homologous challenge, but partial or total lack of crossprotection between isolates has been demonstrated[1,3]. The high level of genetic and antigenic diversity of *Cowdria* has been confirmed recently[4–6]. This will further complicate the development of a recombinant vaccine, which is nevertheless recognized as the most practical solution to the problem in the long term.

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The cytokine network

Cytokines are important mediators of protective and pathological immune responses. An individual cytokine is able to stimulate the production of many others, generating a network that orchestrates the host's immune response to infection. *Cowdria ruminantium* has a profound effect on ECs, including induction of cytokines

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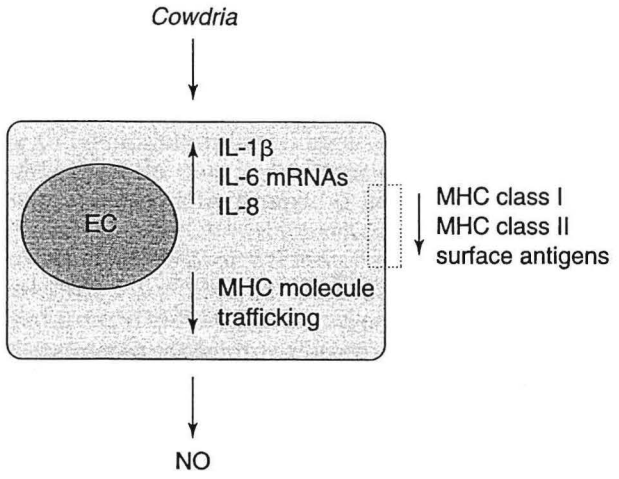

Fig. 1. Pleiotropic effects of *Cowdria ruminantium* on endothelial cells (ECs). *Cowdria ruminantium* elicits *de novo* synthesis of interleukin 1β (IL-1β), IL-6 and IL-8 mRNA in bovine brain microvessel ECs. Interferon γ (IFN-γ)-induced major histocompatibility complex (MHC) class II and constitutive MHC class I surface expression are strongly inhibited upon infection of ECs with *Cowdria*. Part of this effect can be attributed to a disruption in the intracellular trafficking of MHC molecules. Finally, under certain conditions, *C. ruminantium* seems to be able to induce the production of nitric oxide (NO) by infected ECs.

(Fig. 1). *In vitro*, *C. ruminantium* elicits *de novo* synthesis of interleukin 1β (IL-1β), IL-6 and IL-8 mRNA in bovine brain microvessel ECs[7], and this effect is potentiated by interferon γ (IFN-γ). IL-1 and IL-6 can act as co-stimulatory signals for T- and B-cell activation[8]. Therefore, brain ECs, which constitute one of the main targets of *C. ruminantium* in ruminants could contribute to the development of a protective immune response against the pathogen. Paradoxically, uncontrolled or excessive production of these proinflammatory cytokines could contribute to the pathophysiology of heartwater.

Interferons play a major role in protection against intracellular pathogens, including *Cowdria*-related organisms such as *Rickettsia* and *Chlamydia*[9]. The first report of an inhibitory effect of IFN-α and IFN-γ on *C. ruminantium* increased the interest in these cytokines[10]. Cattle that resisted lethal challenge were shown to produce IFN-α whereas animals that died did not[11]. However, even at high concentrations (up to 1000 U ml^{-1}), recombinant IFN-α could not completely prevent the growth of *Cowdria in vitro* in bovine ECs[11], suggesting that other factors were involved in protection. Growing evidence suggests that IFN-γ is one of them. Indeed, there are at least four pathways through which IFN-γ could intervene in protection (Fig. 2). A direct effect on bovine and caprine ECs that renders them unsuitable for *Cowdria* growth was demonstrated for recombinant[10,12] and endogenous IFN-γ produced by concanavalin A-stimulated T cells[13,14]. IFN-γ completely abrogated the growth of *Cowdria* regardless of the strains and EC lines used in these studies. The mechanisms whereby IFN-γ inhibits the growth of *Cowdria* remain to be elucidated. At doses that are low, yet inhibitory for *Cowdria* growth (≤5 U ml^{-1}), IFN-γ neither induced nitric oxide (NO) production by infected ECs[7,14] nor cytotoxicity[12]. However, above 25 U ml^{-1}, IFN-γ treatment increased NO production by infected ECs and also induced apoptosis of these cells as well as non-infected ECs[15], which is difficult to reconcile with a role in protection. Nevertheless, *in vivo*, combinations of *Cowdria* and IFN-γ in synergy with other cytokines might result in NO production by ECs and/or monocytes without affecting vascular wall integrity. In that case, NO could be beneficial for the host because it significantly reduces the viability and infectivity of the extracellular form of *Cowdria*[15].

Another important role for IFN-γ is the upregulation of major histocompatibility complex (MHC) class I and II expression on a wide variety of cells including ECs[16]. However, both IFN-γ-induced MHC class II and constitutive MHC class I surface expression are strongly inhibited upon infection of ECs with *Cowdria* in a dose-dependent manner[12,17]. Part of this inhibition is due to a disruption in the intracellular trafficking of MHC molecules, as shown by permeabilization studies, although at high infection rates inhibition of synthesis also seems to occur[17]. Nevertheless, *Cowdria*-infected ECs were shown recently to induce MHC class II-restricted proliferation of peripheral blood mononuclear cells (PBMCs) from cattle immunized by infection and treatment, provided they were treated with T-cell growth factor (TCGF) to upregulate MHC class II expression[18]. These conflicting results might be attributed to subtleties of experimental protocol. In the first studies, ECs were treated with IFN-γ together with or just after infection; in others, ECs were incubated for 48 h with TCGF before infection. Monocytes have also been shown to present *Cowdria* antigens in association with MHC class II molecules[18,19]. Although these cells are not a privileged target (cells in which *Cowdria* replicates), extracellular *Cowdria* are found in the blood and, therefore, are available for phagocytosis by monocytes. Thus, upregulation of MHC class II expression by IFN-γ on monocytes is likely to favour presentation of *Cowdria* antigens to the immune system.

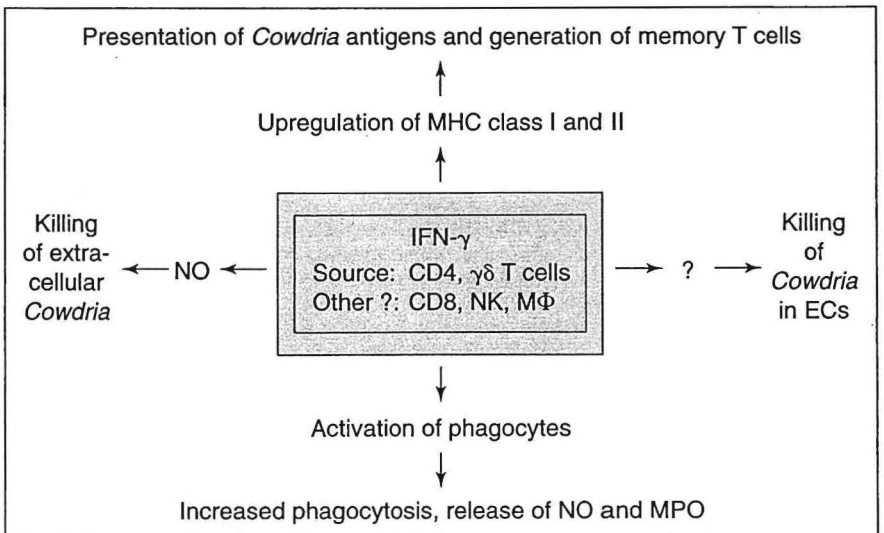

Fig. 2. Putative roles of interferon γ (IFN-γ) in protection against *Cowdria ruminantium*. ECs, endothelial cells; MΦ, monocytes/macrophages; MHC, major histocompatibility complex; MPO, myeloperoxidase; NK, natural killer cells; NO, nitric oxide.

Table 1. T-cell responses elicited in immunized animals by recombinant and native proteins of *Cowdria ruminantium*[a]

Cowdria proteins	T-cell responses	Cytokine expression	Ref.
MAP-1	γδ in animals immunized with killed *Cowdria*	ND	34
	$CD4^+$ in animals immunized by infection and treatment	Th1 type	35
MAP-1B	γδ in animals immunized with killed *Cowdria*	ND	34
MAP-2	γδ in animals immunized with killed *Cowdria*	ND	34
	γδ in animals immunized by infection and treatment	Th1 type	35
22–32 kDa FPLC fraction	$CD4^+$ in animals immunized with killed *Cowdria*	IFN-γ	28

[a] Abbreviations: FPLC, fast performance liquid chromatography; IFN, interferon; MAP, major antigenic protein; ND, not determined; Th1, T helper 1.

Finally, IFN-γ has a profound effect upon monocytes and neutrophils, including increased phagocytosis and, in combination with other cytokines, such as tumour necrosis factor α (TNF-α) and/or bacterial phagocytosis, release of reactive oxygen intermediates, NO[20] and lysosomal enzymes, such as myeloperoxidase (MPO)[21]. Recombinant MPO has been shown to confer protection in a murine model of cowdriosis[22], probably by activating intraperitoneal macrophages to kill *Cowdria*. Although that mechanism is not transposable to ruminants, MPO might be involved in protection through a direct toxic effect on the extracellular form of *Cowdria* and/or indirectly by triggering IFN-α production by monocytes[23].

The role of antibodies

Infection with *Cowdria* elicits antibody responses in ruminants that recover from the disease. However, antibody levels do not correlate with protection or duration of immunity[24]. In some instances[25], hyperimmune sera were shown to neutralize the infection *in vitro*, whereas in others no significant effect was observed[26]. Transfer of immune serum or gamma globulins failed to protect animals or even modify the course of the disease[25,27]. Although these results are not sufficient to exclude the existence of protective antibodies, they underline the limitations of using immune sera to study protective mechanisms. Nevertheless, the potential role of antibodies in opsonization, complement-mediated killing and antibody-dependent cell-mediated cytotoxicity deserves further investigation.

Crossprotection studies have shown the existence of fully, non- or partially crossprotective isolates, indicating that antigens responsible for protection are polymorphic. The use of isolate-specific antisera adsorbed on another, partially crossprotective isolate of *Cowdria* to identify polymorphic immunodominant antigens by ELISA and immunoblots[6] revealed polymorphic proteins of 26–27 kDa and 23 kDa. Interestingly, these proteins fall into the size range shown to induce proliferation of *Cowdria*-specific, IFN-γ-producing, $CD4^+$ T-cell lines derived from immune cattle[28].

Cell-mediated immunity

Because *Cowdria* is an obligate intracellular bacterium, cell-mediated immune responses are expected to play a pivotal role in protection. The capacity of $CD4^+$ T cells to proliferate and produce IFN-γ in response to *Cowdria* lysates and to *Cowdria*-primed autologous monocytes was demonstrated in cattle immunized with killed *Cowdria*[19]. MHC class II-restricted proliferation was also observed with PBMCs obtained from cattle immunized with live *Cowdria*, but only in response to infected, autologous ECs and *Cowdria*-primed monocytes, and not to killed organisms[18]. Thus, during infection with live virulent *Cowdria*, T-cell responses might be preferentially directed at certain epitopes expressed by infected cells but absent from the extracellular form of the organism. γδ T cells are also induced *in vitro* in cattle undergoing immunization with live *Cowdria*[18]. These cells proliferate in response to autologous and heterologous infected ECs and monocytes (thus in an MHC-unrestricted manner) and produce IFN-γ[18].

Contrary to expectations, studies in goats and cattle did not reveal major differences in circulating $CD4^+$ and γδ T cells between naïve and vaccinated animals undergoing challenge, apart from a less severe depletion of $CD4^+$ T cells in animals that survived (D. Martinez, PhD Thesis, University of Utrecht, 1997). Moreover, a spectacular rise in $CD8^+$ T cells occurred in all animals vaccinated with killed *Cowdria* but late after challenge, at a time when all naive controls had already died (D. Martinez, op. cit.). These results suggest that other mechanisms of protection take place before a $CD8^+$-mediated response is triggered. Additional work is needed to investigate the functions of $CD8^+$ T cells and determine whether or not they can protect. Reports that similar changes in $CD8^+$ T cells also occur in a mouse model of cowdriosis, in which the protective capacity of immune $Lyt2^+$ T cells was demonstrated by adoptive transfer experiments[29,30], is supportive evidence.

Antigens involved in humoral and cell-mediated immunity

Among all proteins of *Cowdria* recognized by immune sera, only four have been cloned and fully sequenced. These are major antigenic proteins MAP-1 (Ref. 31) and MAP-2 (Ref. 32), and heat-shock proteins GroES and GroEL[33]. They are conserved among isolates but, in the case of MAP-1, studies have demonstrated high genetic and antigenic diversity[4,6]. MAP-2 induces proliferation of γδ T cells *in vitro* in cattle immunized with both killed and live *Cowdria* (Table 1). Upon stimulation, MAP-2-specific γδ T-cell lines strongly express IFN-γ, IFN-α and TNF-β mRNA, but express little IL-2 and no IL-4 or IL-10, which is consistent with a T helper 1 (Th1)-type response[35]. By contrast, $CD4^+$ T-cell lines specific for MAP-1 and expressing Th1-type cytokines were generated from animals immunized with live, but not killed, *Cowdria* (Table 1). In animals immunized with killed organisms, MAP-1 and MAP-1B (MAP-1B is a specific immunogenic region of MAP-1)[36] repeatedly induced γδ T cells to proliferate. In addition, $CD4^+$ T-cell lines derived from cattle immunized with killed organisms by weekly restimulation of PBMCs *in vitro* with *Cowdria* lysates did not recognize any of the recombinant proteins[34]. Partial fractionation of *Cowdria* proteins by gel filtration and fast performance liquid chromatography (FPLC) showed that soluble antigens

capable of inducing $CD4^+$ T-cell lines to proliferate and produce IFN-γ have a molecular mass of 22–32 kDa[28]. The discrepancy between the results obtained by the two methods of immunization probably reflects differences in the type of immunity that was elicited and the nature of the antigenic determinants involved in these responses.

MAP-1 is the only well-characterized antigen shown so far to be partially protective in mice. Intramuscular injection of a DNA vaccine containing the *map1* gene protected 23–88% of mice against lethal challenge[37]. However, immunization with denatured MAP-1, excised from SDS-PAGE gels and emulsified in complete Freund's adjuvant, did not protect goats and sheep, despite high antibody titres before challenge[38]. Unfortunately, too little information is available on cellular responses to vaccination to allow comparison between the two studies at this stage.

Concluding remarks

The little we know about cytokines in heartwater suggests a high level of complexity, with several factors exerting a variety of effects that can result in resistance but also in disease exacerbation. For example, IFN-γ is a very potent inhibitor of *Cowdria in vitro*, but can also be highly cytotoxic for infected and uninfected ECs. A net positive or negative effect of this cytokine most likely depends on the level and the timing of its production during infection. Thus, early induction of IFN-γ can be expected to control the infection, whereas in late-stage clinical disease, IFN-γ could have detrimental consequences. Of course, most of the information on cytokines is derived from *in vitro* studies, so that the relevance of these results to the *in vivo* situation remains to be defined. No doubt, studies of murine models of cowdriosis, especially gene knockout mice, will be very informative, but great care should be taken in extrapolating these results to ruminants. Although the roles of IFN-γ and IFN-α have not been demonstrated conclusively *in vivo*, their inhibitory effect on the growth of *Cowdria* is well documented. Therefore, antigens capable of inducing the production of these cytokines by immunocompetent cells have clear potential in the development of a recombinant vaccine against heartwater. The source of IFN-γ and IFN-α is not confined to a single cell type. Natural killer cells and macrophages are also capable of producing these cytokines, and play a crucial role in innate immunity to intracellular pathogens. In addition, the possible contribution of infected neutrophils in cytokine production is virtually unknown. Future studies are needed to address the potential role of these cells in immune responses to *Cowdria*.

Although our knowledge of protective immunity to *C. ruminantium* is still fragmentary, significant advances are being made in understanding the mechanisms underlying antibody and cell-mediated immune responses to this pathogen. Differences exist between immunization by infection and treatment or with killed *Cowdria*, as illustrated by studies using MAP-1. In the first case, MAP-1 is serologically immunodominant and induces the proliferation of $CD4^+$ T cells, whereas, in the second, antibodies as well as $CD4^+$ T cells might be preferentially directed against other proteins. In both models, T helper cells are capable of producing IFN-γ in response to *Cowdria* antigens. In addition, γδ T cells specific for *Cowdria*-infected ECs and for MAP-1 and MAP-2 are also induced during immunization and have been shown to produce Th1-type cytokines. The availability of *Cowdria*-specific T-cell lines will enable us to identify components of the agent that provoke these responses and will, therefore, be of relevance in the development of a subunit vaccine against the disease. Finally, although *Cowdria*-specific $CD8^+$ T cells have not yet been reported, studies performed *in vivo* in mice and ruminants suggest a role of these cells in protection. Immune cell transfer experiments are needed to assess the relative contribution of the different T-cell subpopulations in protective immune responses. However, an effective vaccine need not necessarily mimic immune responses implicated in protection under natural circumstances. In other words, artificial manipulation of the host's immune system using appropriate antigen delivery systems might result in protective responses against antigens that might not be involved in protective immunity in animals immunized with the whole organism. Diverse antigen delivery systems, such as capripox, *Salmonella* and naked DNA, are currently being evaluated using expression libraries and available genes encoding immunodominant proteins.

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Cytometry 49:49-55 (2002)

Original Articles

Evaluation of Several Flow Cytometric Assays for the Analysis of T-Cell Responses in Goats

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Background: Flow cytometry (FCM) provides an alternative to radioactive methods for the analysis of T-cell responses. However, a comparative study of common FCM assays in an outbred ruminant model is lacking, which motivated this work.

Methods: Goats immunized with the obligate intracellular bacterium *Cowdria ruminantium*, inactivated and emulsified in oil-based adjuvants, were used as a model to study T-cell recall responses in vitro. FCM-based methods to measure *Cowdria*-induced lymphoblastogenesis, DNA synthesis, and interleukin-2 receptor (IL-2R) expression by T-cell subsets were compared.

Results: IL-2R expression was the most sensitive and reliable method provided that the number of molecules per cell was analyzed and not simply the percentage of positive cells of a given phenotype. Despite high background due to adjuvant and low proliferation, this method could detect antigen-specific activation of immune $CD4^+$ and $CD8^+$ T cells.

Conclusions: FCM-based measurement of lymphoblastogenesis and DNA synthesis are not the most appropriate methods to analyze T-lymphocyte activation during vaccination of outbred animals. On several occasions, analysis of IL-2R expression was the only assay capable of discriminating between vaccinated and naive animals in this model. Cytometry 49:49-55, 2002.
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Key terms: T-cell recall responses; lymphoblastogenesis; DNA synthesis; interleukin-2 receptor expression; outbred animal model; goat; obligate intracellular bacterium; *Cowdria ruminantium*; killed vaccine

T-cell activation is a prerequisite to effector function; therefore, studies of the kinetics of T-cell activation during infection and immunization generally precede more complicated functional analysis. This is even truer for killed and recombinant vaccines because of low immunogenicity. More over, strong adjuvants such as complete and incomplete Freund's adjuvants and analogues have been shown to possess intrinsic immunomodulatory activity resulting in T-cell activation in the absence of any antigens (1,2) (present study) and thus may complicate the study of cellular responses induced during vaccination. More recently, nucleic acid vaccination has attracted considerable interest because of its ability to generate a broad range of immune responses without the use of conventional adjuvants (3). Nevertheless, the challenge ahead with this method will be to increase immune responses to lower the amount of DNA needed in animals other than mice, which presently prohibits its use for livestock vaccination (4). For these reasons, the development of sensitive and reliable methods to measure T-cell activation in outbred animals is of considerable importance.

Our group is involved in the development of efficient and affordable recombinant vaccines against tropical diseases of ruminants in general and goats in particular because of their importance for rural and peri-urban communities in developing countries. One of our targets is a tick-borne disease called heartwater or cowdiosis and caused by the obligate intracellular bacterium *Cowdria ruminantium* (5). The immune response induced in goats by a protective killed *Cowdria* vaccine (6) is currently being studied, and these animals were used in this study.

T-cell activation is characterized by early biochemical events such as blastogenesis and increased interleukin-2 receptor (IL-2R) expression and DNA synthesis, and these events have been examined with nonradioactive flow cytometry (FCM)-based methods in ruminants (7-9). However, a comparative study of common FCM assays in an

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outbred ruminant model, specifically in goat, is lacking, which motivated this work.

MATERIALS AND METHODS

Animals and Immunization Protocols

Creole goats originating from Les Saintes Islands, a cowdriosis-free region of the Caribbean, were used throughout this study. Six different experiments were carried out over a 4-year period. Immunization with killed *Cowdria* organisms was always done with subcutaneous injections of 1 ml of in vitro grown elementary bodies (EBs) inactivated overnight with 0.1% sodium azide and mixed 1:1 in oil-based adjuvant (6). In the first experiment (group A: animals 95203, 95209, and 95213), animals received 250 μg of killed *Cowdria* emulsified in ISA50 (Seppic, Paris, France), followed 2 and 12 months later by similar booster inoculations. In the second experiment (group B: animals 9906, 9908, and 95206), we investigated the effect of increasing doses of *Cowdria* antigens on T-lymphocyte recall responses in vitro. Animals received 40 μg of killed *Cowdria* in ISA50, followed 6, 18, and 29 weeks later by 40, 100, and 300 μg, respectively. In experiment C, three animals (9713, 9715, and 9901) received 200 μg of killed *Cowdria* in complete Freund's adjuvant (CFA; Sigma, Saint-Quentin, France) and again 8 weeks later, but in incomplete Freund's adjuvant (IFA). In addition, two control animals (9802 and 9820) received CFA in phosphate buffered saline (PBS) and IFA in PBS for control purposes. Animals (0108, 0113, and 95224) from group D received the same vaccination regimen as group C, but we used ISA50 instead of CFA/IFA. Two control animals received, in parallel, PBS plus ISA50. The Gardel strain of *Cowdria* isolated in Guadeloupe (10) was used in experiments A–D. In experiment E (9815, 9913, and 9920), the vaccination protocol in experiment D was used, but with a different strain of *Cowdria*, the Welgevonden strain, originating from South Africa (11). In the last experiment (group F), the effect of subcutaneous injections of 1 ml of PBS alone (i.e., without adjuvant) was studied in four control goats.

Stimulation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by density gradient centrifugation on Histopaque (Sigma). After washing in Alsever's solution to remove platelets, cells were resuspended in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 5×10^{-5} M 2-mercaptoethanol. Fresh PBMC were cultured for 4 days at a density of 1.5×10^6 cells/ml in total volumes of 200 μl/well and 2 ml/well of 96-well and 24-well plates, respectively. *Cowdria* lysates were prepared from in vitro cultures in bovine endothelial cells, as described previously (12). Briefly, *Cowdria* EBs released in the supernatant of cells undergoing lysis were purified by differential centrifugation and washed twice in 0.1 M PBS. After the final wash, EBs were resuspended in PBS, homogenized by passing three times through a 30-gauge needle, lysed by two freeze–thaw cycles at −20°C, aliquoted and stored at −20°C until use. Lysates of uninfected endothelial cells also were prepared for control purposes. Antigens (Ag) were used at a final concentration of 1 μg/ml. Concanavalin A (ConA) was used as a positive control at a final concentration of 5 μg/ml. For studies on DNA synthesis, 0.66 μg/ml of colchicine (Sigma) was added to the cultures on day 3 to prevent mitosis.

Flow Cytometry

Two to three fluorescent parameters analysis was performed on a FACSCalibur flow cytometer equipped with CellQuest 3.01 (Becton Dickinson, San Jose, CA, USA). A minimum of 5×10^5 PBMC obtained from stimulated and control cultures were added to each well of a 96-U-well microtiter tray to allow analysis of 5,000–10,000 events for each T-cell subset. For DNA staining, six times as many cells were required due to losses after the fixation and permeabilization steps. The phenotype of responding T lymphocytes was analyzed by using the following lineage-specific mouse monoclonal antibodies: GC50 against CD4 (immunoglubulin M, IgM) (13), 7C2 against CD8 (IgG2a) (14), and CC15 against WC1 found on all γδTCR + PBMC (IgG2a) (15). Normal mouse serum (Sigma) was used to evaluate nonspecific biding and to set markers delineating positive populations. Single fluorescence-stained samples were used to optimize compensation of signals.

Lymphoblastogenesis. The forward (FSC) and right-angled (SSC) light scatter characteristics generated by the flow cytometer provide an estimation of cell size and granularity, respectively. Using a gate set on a FSC versus SSC dotplot, dead cells and a majority of B lymphocytes were excluded from the analysis to focus on viable T lymphocytes. Changes in size of the different T-cell subsets were analyzed on fluorescence versus FSC dotplots after cell-surface phenotyping, as described below.

IL-2R surface expression. Mouse mAb CACT116A (IgG1), which recognizes the α chain of bovine and caprine IL-2R was used in this study (VMRD, Pullman, WA, USA). Briefly, after washing in PBS containing 2% heat-inactivated horse serum, 0.1% NaN3, and 10 mM ethylenediaminetetra acetic acid (FACS medium), PBMC were resuspended in 100 μl of optimally diluted CACT116 and lineage-specific mAbs. After 30 min at 4°C, cells were washed three times in FACS medium and stained with a cocktail containing the following fluorochrome-conjugated secondary antibodies (Caltag, San Francisco, CA, USA): goat anti-mouse IgG1 coupled to Tricolor, goat anti-mouse IgG2a coupled to fluorescein isothiocyanate, and goat anti-mouse IgM coupled to phycoerythrin.

DNA content. DNA increase in T-cell subsets was analyzed by staining with mAbs against surface antigens followed by propidium iodide uptake in permeabilized cells. Surface phenotyping was performed as above except that primary mAbs were used separately and only fluorescein isothiocyanate–coupled goat anti-mouse Igs were used as conjugates. DNA staining was done as described previously (16), with minor modifications (17).

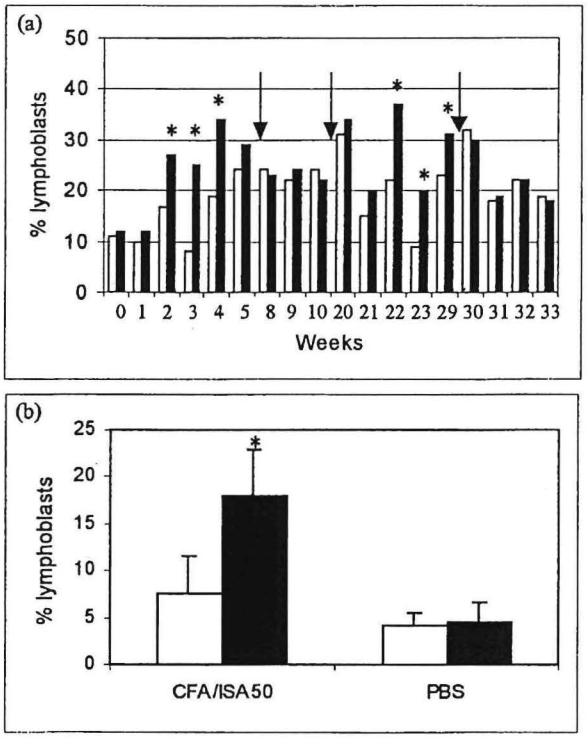

FIG. 1. a: Kinetics of *Cowdria*-induced T-lymphocyte blastogenesis during vaccination with killed *Cowdria* emulsified in oil adjuvant. Results are expressed as the percentage of blasts after 4 days of culture with (black) or without (white) *Cowdria* antigens. Data are shown for one animal in group B. Arrows indicate booster injections. b: Effect of oil adjuvant on the background blastogenesis of PBMC after 4 days of culture with medium alone. A comparison is shown between PBMC collected at day 0 (white) and 3 weeks after inoculation (black) with oil adjuvants (CFA/ISA50) or PBS alone (PBS). Results are means and standard errors of four animals in each group. Asterisks indicate significant difference between black and white histograms.

Briefly, after cell-surface phenotyping, PBMC were washed twice in Hank's balanced saline solution (HBSS) with Mg^{2+} and Ca^{2+} (Sigma), resuspended in 50 μl of HBSS to which 150 μl of 70% ethanol was progressively added, and incubated for 30 min at 4°C. After one wash in HBSS, cells were incubated for 5 min at room temperature in 100 μl of HBSS and 50 μl of phosphate citric acid buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.8). This step was necessary to avoid the presence of a shoulder in the G0/G1 peak due to apoptotic cells. After centrifugation, cells were transferred to round-bottom tubes and incubated for 30 min in the dark at 4°C in 1 ml of HBSS containing 20 μg/ml of propidium iodide and 5 Kunitz units of the DNase-free RNase A (Sigma). Acquisition of samples was done by using a gate set on FL2-W versus FL2-A dotplots to include only singlets. Percentages of cells within G0/G1 (2n), S (between 2n and 4n), and G2/M (4n) phases of the cell cycle were obtained for each T-cell subset from FL1 versus FL2-A dotplots.

Reproducibility of FCM assays. On several occasions, standard deviations of *Cowdria*-induced and ConA-induced responses were estimated for each T-cell subset from triplicate tests. Standard deviations of the percentages of lymphoblasts and T cells positive for IL-2R or within G0/G1, S, and G2/M phases of the cell cycle were always below 5. Thus, values above 5% were considered positive. For results expressed as percentage of increase in the mean intensity of fluorescence (MIF), values above 20% were considered positive.

Statistical Analysis

Differences between mean values were analyzed for significance by the Wilcoxon-Mann-Whitney nonparametric test. $P < 0.05$ was considered significant.

RESULTS

Lymphoblastogenesis

An increase in lymphoblasts was observed as soon as 2 weeks after vaccination (Fig. 1A), with a peak at approximately week 3 or 4 depending on the animal. However, background response (i.e., response to medium alone)

Table 1
Phenotype of Lymphoblasts After 4 days of Culture With or Without Cowdria Antigens and Uninfected Cell Antigens

Animal number	Total lymphoblasts[a]				Phenotype of lymphoblasts[b]					
					CD4					
	NoAg[c]	CAg	CellAg	ConA	CAg	CellAg	CD8		WC1	
Group C										
9713	24	34[d]	23	72[d]	24[d]	6	32[d]	25	20	29
9715	15	27[d]	14	71[d]	20[d]	6	23[d]	14	6	11
9901	14	36[d]	12	79[d]	19[d]	5	24	22	7	12
Group D										
95224	20	53[d]	36	87[d]	45[d]	32	23	21	4	6
0108	29	57[d]	45	86[d]	40	35	32	28	11	13
Group E										
9815	18	33[d]	23	65[d]	26	22	19	15	13	20
9913	22	42[d]	30	70[d]	37[d]	25	25[d]	15	8	7

[a]Percentage of peripheral blood mononuclear cells.
[b]Percentage of a given phenotype within the lymphoblast population.
[c]NoAg, medium only; CAg, *Cowdria* whole lysate; CellAg, uninfected cell antigens; ConA, concanavalin A.
[d]*Cowdria* effect was significantly above that of the CellAg control (>5%).

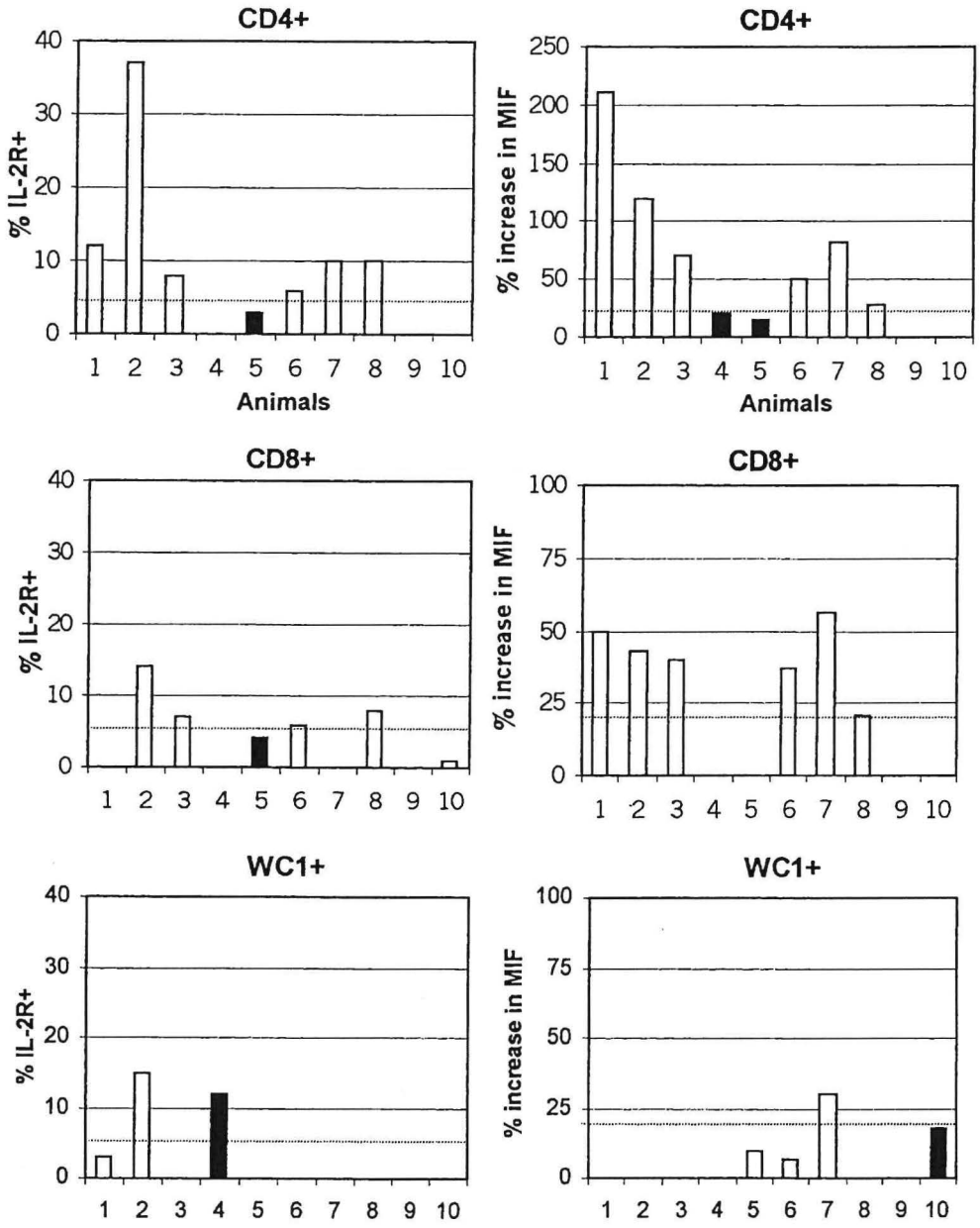

FIG. 2. Discrimination between vaccinated (white bars) and control (black bars) animals by use of IL-2R expression on T-lymphocyte subsets. PBMC were collected 3 weeks after vaccination and incubated for 4 days with or without antigens. The highest background (i.e., no antigen or cell antigen controls) deducted from these data showed only the net effect of *Cowdria* antigens. Results of two different experiments (groups C and D) are shown. Results are expressed as the percentages of IL-2R$^+$ cells of a given phenotype (left) and of the increase in MIF, which is an estimation of the number of IL-2R molecules per cell (right). Percentages above dotted lines were considered significant (see Materials and Methods for reproducibility of FCM).

also increased progressively. After booster injections, significant blastogenesis of PBMC was seldom observed and never above the level of the first injection (Fig. 1A). Similar observations were made for all animals from groups B and C. Part of the background response of PBMC after in vitro culture can be attributed to an adjuvant effect (Fig. 1B).

The phenotype of responding PBMC was analyzed by FCM 2 (group C) and 3 (groups C–E) weeks after the first inoculation and 1 (groups C and E) or 2 (group D) weeks after the booster inoculation. Analysis at week 3 after vaccination produced the best results and is summarized in Table 1. *Cowdria* Ags induced a significant blastogenesis of PBMC in seven of nine animals tested. Increases in

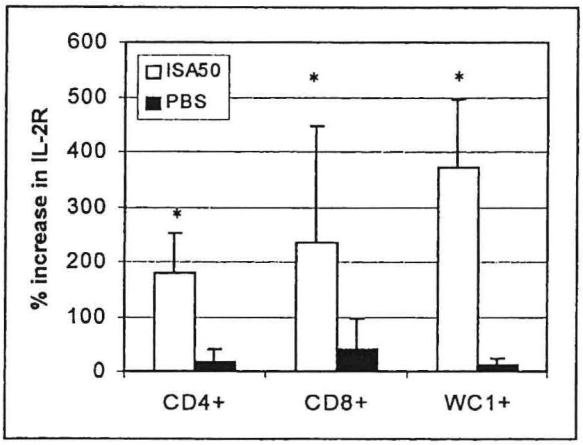

FIG. 3. Effect of adjuvant on the background expression of IL-2R on T-lymphocyte subsets. PBMC were collected 3 weeks after subcutaneous inoculation of ISA50 in PBS (white bars) or PBS alone (black bars) and incubated in vitro for 4 days in medium alone. Results are means and standard errors of four animals and expressed as the percentage increase between days 0 and 4 of MIF. Asterisks indicate significant differences ($P < 0.05$) between black and white histograms.

$CD4^+$, $CD8^+$, and $WC1^+$ lymphoblasts were observed on five, three, and zero occasions, respectively. However, this was generally not accompanied by an enrichment of cultures in a given subset. There was no measurable effect of *Cowdria* Ags on T lymphocytes obtained from naive controls (data not shown).

IL-2R Expression

In general, the effect of *Cowdria* Ags on IL-2R surface expression by T-cell subsets was more evident when numbers of IL-2R molecules per cell (MIF) were analyzed instead of percentages of $IL\text{-}2R^+$ cells (Fig. 2). This was particularly true for $CD8^+$ T cells that showed increases in MIF in five of six animals in comparison with only three of six animals when $IL\text{-}2R^+$ cells were considered. Activation of $CD4^+$ T cells was observed for all animals. In contrast, increases of IL-2R expression by $WC1^+$ T cells was observed only occasionally for vaccinated and naive animals (Fig. 2). In experiment E, *Cowdria* Ags increased IL-2R expression on $CD4^+$ and $CD8^+$ cells in two of three vaccinated animals (data not shown). Differences between mean MIF values of vaccinated and control animals from experiments C, D, and E were significant ($P < 0.05$) for $CD4^+$ and $CD8^+$ T lymphocytes. Booster injections did not increase T-cell responses to *Cowdria* Ags (data not shown).

As seen with blastogenesis, there was a substantial activation of all T-cell subsets in the absence of stimuli, and this background response could be attributed to an adjuvant effect (Fig. 3). $WC1^+$ T lymphocytes showed the highest background increase of IL-2R expression, although these cells were the least activated (i.e., lower MIF values) of all subsets at day 0 (not shown). Background activation of WC1 cells was induced by ISA50 and CFA (Fig. 4).

DNA Synthesis

The effect of *Cowdria* Ags on the DNA content of T lymphocytes was analyzed with the use of PBMC collected 3 weeks after the first inoculation (group D) and after the last booster injection (groups A and B). DNA synthesis in response to *Cowdria* Ags was observed in PBMC of three of eight animals tested, but active proliferation (i.e., cells in G2/M phase of the cell cycle) was evident in only two (Fig. 5 and Table 2). In contrast, active proliferation of PBMC in response to ConA was detected in all animals (data not shown). When T-cell subsets were analyzed for DNA content, increased DNA synthesis was found in additional vaccinated animals but only for $CD4^+$ T cells and in the absence of active proliferation (Table 2).

DICUSSION

As expected with outbred animals, T-cell recall responses after vaccination with killed *Cowdria* organisms were highly variable between individuals. Low, medium, and high responders were observed with all methods

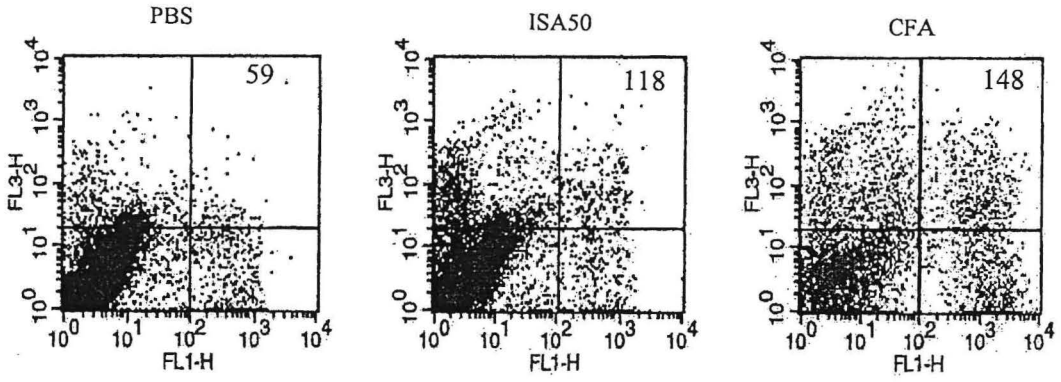

FIG. 4. Effect of ISA50 and CFA on the activation of goat $WC1^+$ T lymphocytes after 4 days of incubation in medium alone. PBMC were collected 3 weeks after subcutaneous inoculation with PBS alone, ISA50 in PBS, and CFA in PBS. The MIF values for IL-2R (FL3, y axis) of $WC1^+$ cells (FL1, x axis) are presented in the upper right quadrant of each plot for one animal in each group. All MIF values at day 0 were near 44 ± 6 (mean ± standard error).

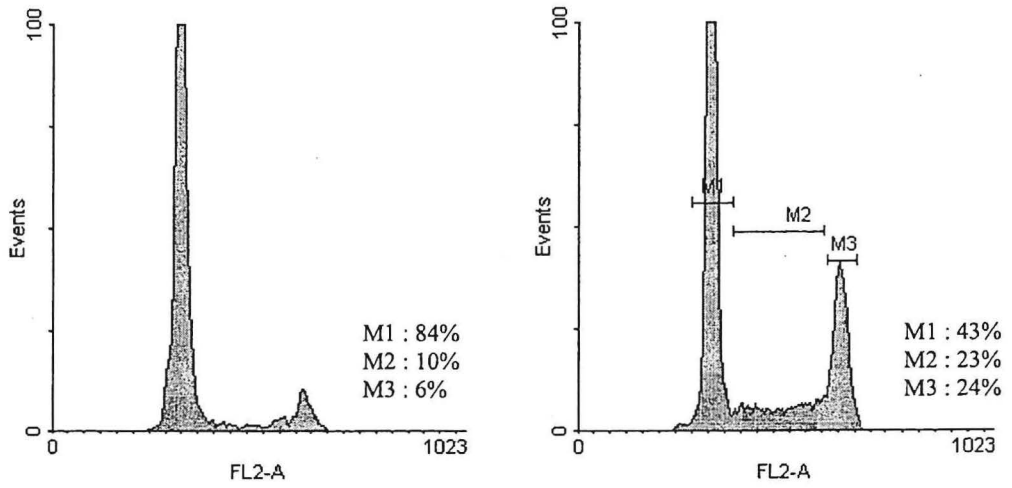

FIG. 5. Cell cycle analysis of PBMC obtained from animal 95209 and incubated for 4 days with uninfected cell (a) and *Cowdria* (b) antigens. DNA content is presented as FL2 on the x axis, and the number of events is on the y axis. The percentages of cells in G0/G1 (M1), S (M2), and G2/M (M3) phases of the cell cycle are presented in each histogram.

used. Nevertheless, a similar type of Ag-driven response was detected in vitro with each method and was characterized by a predominant effect on CD4, less activation of CD8, and a negligible effect on $WC1^+$ T cells. There was no correlation between lymphocyte subset concentration and activation after in vitro culture.

The detection of T-cell responses in this goat model was complicated by a high background activation of primed and unprimed lymphocytes due to adjuvant. The use of autologous heat-inactivated serum instead of fetal calf serum did not significantly lower the background (data not shown). In addition to a masking effect, it is possible that this background activation negatively affects the response to *Cowdria* Ags. The following observations support this hypothesis: (a) IL-2R expression on $CD4^+$ and $CD8^+$ T cells increased in response to *Cowdria* Ags, but these cells did not actively proliferate, and on several occasions DNA synthesis was not detected; and (b) the effect of adjuvant was maximal on $WC1^+$ T lymphocytes, which have been shown in other models to exert a suppressive effect on $CD4^+$ T cells (18-20).

The previously described (21) whole-blood method for quantification of IL-2R expression on caprine lymphocytes also was tested in our model (not shown). Although a strikingly lower background was observed, it did not result in a significant increase of *Cowdria*-induced responses. However, degranulation of neutrophils was observed in the presence of *Cowdria* Ags and mitogens and may have affected T-cell responses through the release of toxic products (22).

In summary, our results clearly show (Table 3) that the IL-2R assay is the most appropriate FCM-based method for the analysis of T-cell responses in goats. On several occasions, the IL-2R assay was the only method capable of discriminating between vaccinated and naive animals, characterized by high background due to adjuvant and low proliferation. This method will be instrumental in the search of adjuvants and Ag delivery systems that can

Table 2
Detection of Cowdria-Induced DNA Synthesis in Goat T Lymphocytes by Flow Cytometry

Animal number	DNA increase[a]					
	PBMC		$CD4^+$		$CD8^+$	
	S	G2/M				
Group A						
95209	13[b]	18[b]	22[b]	16[b]	17[b]	12[b]
95213	13[b]	7	9[b]	9[b]	13[b]	9[b]
95203	2	2	10[b]	3	2	2
Group B						
9908	2	1	7[b]	3	6	−5
95206	4	2	18[b]	2	5	−4
Group D[c]						
95224	8[b]	2	8[b]	2	5	3
0108	2	0	3	0	3	0
0113	3	2	4	0	3	1

[a]Percentage of cells in S and G2/M phases above that of control cells in medium alone and uninfected cells.
[b]significant increase (>5%).
[c]Animals 95224, 0108, and 0113 correspond to animals 6, 7, and 8 in Figure 2.

Table 3
Success of FCM Assays in Detection of T-Cell Responses in Goats

Assays	T-cell subset[a]	
	$CD4^+$	$CD8^+$
Blastogenesis	5/9	3/9
IL-2R expression	8/9	7/9
DNA synthesis	6/8	2/8

[a]Positive animals/total number of animals tested.

increase T-cell recall responses of outbred animals after vaccination with recombinant vaccines.

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Protective killed *Ehrlichia ruminantium* vaccine elicits IFN-γ responses by CD4+ and CD8+ T lymphocytes in goats

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Abstract

Interferon gamma (IFN-γ) is considered as a key mediator of protective cell-mediated immunity against intracellular pathogens in general, and against *Ehrlichia ruminantium*, the causative agent of tick-borne heartwater disease of ruminants, in particular. However, the source of this important cytokine in animals immunized against *E. ruminantium* remains largely unknown. We have analyzed in goats protected by vaccination with a killed *E. ruminantium* vaccine, the potential of individual, genuine (i.e., non-cloned), T cell subsets to produce IFN-γ after antigenic recall in vitro. In all vaccinated but none control animals, *E. ruminantium*-induced IFN-γ secretion was observed in 24 h stimulated blood. Flow cytometric analysis of stimulated peripheral blood mononuclear cells (PBMCs) collected after each vaccine inoculation indicated that immune CD4+ and CD8+ T cells contribute to the same extent to the production of IFN-γ, while WC1+ T cells are less important. This was confirmed by blocking the secretion of IFN-γ with anti-classes I and II major histocompatibility complex antibodies. Blocking experiments also suggest that CD8+ need the help of CD4+ T cells in order to produce IFN-γ. Thus, this work underlines the key role of CD4+ T cells in the production of IFN-γ by immune goat PBMC. It also describes, for the first time in ruminants, *E. ruminantium*-specific CD8+ effector T cells. Since CD4+ and CD8+ T cells collectively contribute to the production of IFN-γ in most vaccinated animals, and since these responses are associated with protection, it may be that a recombinant vaccine will need to incorporate *E. ruminantium* antigens capable of driving both responses.

Keywords: *Erhlichia ruminantium*; Killed vaccine; IFN-γ; Caprine T lymphocytes

1. Introduction

Ehrlichia ruminantium (ex *Cowdria ruminantium*) is a tick-borne intracellular bacterium that causes heartwater, an economically important infectious disease of ruminants in sub-Saharan Africa and in certain Caribbean Islands (Perreau et al., 1980; Provost and

Abbreviations: CI, calcium ionophore; PE, phycoerythrin; TC, tricolor

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Bezuidenhout, 1987). The methods of vaccination used so far in ruminants are: (i) infection with live bacterium and treatment with tetracycline (Van der Merve, 1987); (ii) inoculation of attenuated bacteria (Jongejan, 1991); (iii) inoculation of inactivated organisms in oil-based adjuvants (Mahan et al., 1995; Martinez et al., 1994; Totté et al., 1997). These methods have major drawbacks and there is a general agreement that a recombinant vaccine would be the most practical and affordable solution (Mahan et al., 1999; Totté et al., 1997). A better understanding of immune mechanisms responsible for protection would facilitate the development of such a vaccine.

Growing evidence suggest that IFN-γ plays a pivotal role in protection against *E. ruminantium*. In vitro, a direct effect of IFN-γ on bovine and caprine endothelial cells that renders them unsuitable for *E. ruminantium* growth was demonstrated for recombinant (Totté et al., 1993, 1996) and endogenous IFN-γ produced by concanavalin A-stimulated peripheral blood mononuclear cells (PBMCs) (Mahan et al., 1994, 1996). Other potentially positive effects of IFN-γ have been described (for review see Totté et al., 1999), but the source of this important cytokine in immune animals remains largely unknown. *E. ruminantium*-specific, IFN-γ producing CD4+ and γδ TCR+ T cells lines were obtained from immunized cattle by weekly restimulation of PBMC with *E. ruminantium* lysates and *E. ruminantium*-primed autologous monocytes in vitro (Mwangi et al., 1998; Totté et al., 1997). However, these T cell lines may not be representative of all physiologically relevant events because of the bias introduced by the in vitro culture required to generate them. This bias includes: (i) selection of cells capable to proliferate (i.e., only cells resistant to activation-induced apoptosis are evaluated) (Kabelitz et al., 1993); (ii) possible effect of cytokines produced by activated cells and/or added to promote cell growth (Picker et al., 1995).

The objectives of the present study were: (i) to characterize within a bulk population of immune PBMC, the cell subsets that produce IFN-γ in response to a single, short-time stimulation with *E. ruminantium* antigens; (ii) to confirm in goats, that vaccination with the killed *E. ruminantium* vaccine elicits specific IFN-γ producing T lymphocytes. This was made possible by the use of flow cytometry to simultaneously stain for surface markers and intracellular IFN-γ at a single cell level, thus, allowing determination of the potential of defined T cell subsets to produce the *E. ruminantium*-inhibitory cytokine.

2. Materials and methods

2.1. Vaccine and antigen preparations

E. ruminantium (Gardel strain) organisms were produced in bovine endothelial cells by conventional methods (Martinez et al., 1990). Antigens for immunization were produced as described previously (Martinez et al., 1994), with the following modifications. When the culture showed between 70 and 80% cytopathic effect, the supernatant was collected and cells debris removed by centrifugation at 1800 × *g* for 15 min. The supernatant was further centrifuged at 20,000 × *g* for 30 min and the pellet was resuspended in 0.1 M phosphate-buffered saline, pH 7.1 (PBS). Two more centrifugations at 20,000 × *g* were done to wash the elementary bodies. Protein content was determined by the Bradford assay. The killed vaccine was produced by overnight incubation of *E. ruminantium* elementary bodies with 0.1% sodium azide (Martinez et al., 1994). For in vitro stimulation, a lysate of *E. ruminantium* was prepared by two freeze-thaw cycles at −20 °C. Lysates of uninfected endothelial cells were also prepared for control purposes.

2.2. Animals, vaccination and challenge protocols

Creole goats originating from Les Saintes Islands, a heartwater-free region of the Caribbean, were used throughout this study. Immunization was done by subcutaneous injections of 60 μg of inactivated *E. ruminantium* mixed 1/1 in Montanide ISA50 (SEPPIC, France), followed 1 month later by a booster injection (Martinez et al., 1996). Three animals were used as controls and were inoculated with PBS mixed in Montanide ISA50. Goats were challenged 6 months after boosting by intravenous injection of a virulent culture supernatant containing 2×10^4 live *E. ruminantium* elementary bodies as measured by the Live-dead *Bac*light bacterial viability kit (Molecular Probes, France). All five vaccinated animals survived to virulent challenge without showing any nervous clinical signs. In contrast, two of the three control

animals developed cowdriosis and were euthanized or died within 15 days. Incubation period and time of death correspond to what is observed in the field (Matheron et al., 1987). The experiment was carried out according to the guidelines in the Guide to the Care and Use of Experimental Animals, provided by the French Ministry of Agriculture.

2.3. Evaluation of secreted IFN-γ by ELISA

Heparinized blood was collected from the jugular vein and 200 μl were mixed with 20 μl of antigens at a final concentration of 2.5 μg/ml. Blood incubated without antigens or with non-infected endothelial cells antigens was used as control. For positive control we used blood incubated with phorbol myristate acetate (PMA; 50 ng/ml, Sigma, France) and calcium ionophore A23187 (CI; 1 μg/ml; Sigma, France). Samples were incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator in 96-wells flat-bottomed plates after which plasma supernatants were harvested and assayed with a bovine IFN-γ ELISA kit (Bovigam, CSL, Australia). Results were expressed in percentages of the kit positive control and given by the following formula: positive (%) = 100 × [(sample OD − negative OD)/(positive OD − negative OD)], where OD means optical density at 450 nm. The positive (±6 U/ml) and negative controls are provided with the kit. Blood samples from all animals were collected before and 21 days after vaccination, and 21 days and 6 months after the booster injection. In order to determine the optimal dilutions of monoclonal antibodies (mAb) specific for bovine classes I and II major histocompatibility complex (MHC) molecules for blocking the IFN-γ secretion, preliminary experiments were done before the booster injection. This study was done in parallel with the intracellular labeling, but not in the same animals because the latter technique requires a large amount of blood. Subsequently, blocking experiments were performed in three animals with blood collected 1 week after the booster injection and incubated with *E. ruminantium* antigens and the following monoclonal antibodies (mAb) specific for bovine classes I and class II major histocompatibility complex (MHC) molecules at a final dilution of 1/150: anti-bovine class I MHC, mAb ILA88 (IgG2a) (Toye et al., 1990); anti-bovine class II DR, mAb TH14B; anti-bovine class II DQ, mAb TH81A5 and anti-bovine class II DP, mAb H42A (VMRD, USA).

2.4. PBMC stimulation for intracellular IFN-γ staining

PBMC from three vaccinated and three control goats were obtained from heparinized blood by density gradient centrifugation on Histopaque (Sigma, France). After washing in Alsever's solution to remove platelets, cells were resuspended in RPMI 1640 medium (Sigma, France) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 5×10^{-5} M 2-mercaptoethanol. Fresh PBMC were cultured 2 days at 37 °C in 5% CO_2, at a density of 1.5×10^6 cells/ml, in a total volume of 1.5 ml per well in 24-wells flat-bottomed plates. PBMC were stimulated with *E. ruminantium* lysates at a final concentration of 1 μg/ml. Concanavalin A stimulation (5 μg/ml) was used as a positive control. Negative controls were PBMC incubated without antigens and with uninfected endothelial cells antigens. Fifteen hours before the end of the stimulation, brefeldin A (Sigma, France) was added to the cells at a final concentration of 5 μg/ml to block the secretion of IFN-γ.

2.5. Flow cytometry based evaluation of intracellular IFN-γ in caprine T cell subsets

The method used was based on the technique described by Weynants et al. (1998) with some modifications. After antigenic stimulation, PBMC were collected and washed in PBS with 10 mM EDTA. PBMC were seeded at 2.25×10^6 cells per well in 96-wells round-bottomed plates, washed with PBS and fixed 10 min in 1% formaldehyde. Cells were washed twice in PBS and twice in PBS–10% FCS–0.1% saponine (solution A) before staining with mAb. Simultaneous staining for T cells surface markers and intracellular IFN-γ was done at room temperature during 30 min, with the following mouse antibodies: mAb GC50 (IgM) to identify CD4+ T cells (Bensaid and Hadam, 1991), mAb 7C2 (IgG2a) to identify CD8+ T cells (Maddox et al., 1985) and mAb CC15 (IgG2a) against WC1 found on all circulating γδ TCR+ T cells (Morrison and Davis, 1991).

For IFN-γ we used anti-bovine IFN-γ mAb 7B6 (IgG1) (Weynants et al., 1998). Isotype-matched antibodies for surface cell markers and intracellular staining were always used as control. PBMC were then washed twice with solution A and incubated as above with the following goat anti-mouse fluorochrome-conjugated antibodies (CALTAG, USA): IgM and IgG2a coupled to phycoerythrin (PE) and IgG1 coupled to fluorescein isothiocyanate (FITC). PBMC were washed twice in solution A and twice in PBS and then fixed in formaldehyde as above. On some occasions, three-colors staining of CD4+ and CD8+ T cells and IFN-γ was performed. In these cases, the following goat anti-mouse fluorochrome-conjugated antibodies were used: IgG2a coupled to fluorescein isothiocyanate (FITC); IgM coupled to phycoerythrin (PE); IgG1 coupled to tricolor (TC). Samples were analyzed on a FACScalibur flow cytometer equipped with the CellQuest 3.01 software (Becton Dickinson, San Jose, USA). The typical forward and side scatter lymphocyte gate together with a surface marker gate were set to exclude dead cells and select a given T cell subset for analysis, as previously described (Walravens et al., 2002). Between 15,000 and 30,000 events within this gate were acquired per sample.

It should be noted that, in the presence of *E. ruminantium* antigen and concanavalin A, an increase of up to one log in the fluorescence given by mAb 7B6 was often observed for the entire gated population. However, this was also occasionally seen with the isotype-matched antibody control. Thus, only events above isotype-matched controls and well separated from the main population were considered as IFN-γ+. To facilitate the interpretation of results, cutoff values equivalent to the mean percentage of IFN-γ positive cells in the absence of antigen plus three times the standard deviation, were calculated for each T cell subsets in both vaccinated and control groups. The effect of *E. ruminantium* was considered positive only if above cutoff values. The absence of a signal in non-permeabilized cells confirmed that mAb 7B6 recognizes intracellular IFN-γ and not the extracellular form that may bind the surface of PBMC.

2.6. Statistical analysis

The non-parametric Mann–Whitney *U*-test was used to analyze differences between *E. ruminantium* and control stimulations, and between vaccinated and control goats. A difference was considered to be significant at $P < 0.05$. Statistical analyses were performed using a standard statistical package (StatView, 5.0 for windows, SAS Institute Inc.).

3. Results

3.1. Kinetics of IFN-γ production during vaccination

Whole blood collected before and during vaccination was incubated 24 h with or without *E. ruminantium* antigens and IFN-γ secretion was measured by ELISA in plasma supernatant. Whole blood was chosen instead of PBMC since previous work (Esteves et al., 2002) indicated that high background responses occasionally occur with the latter. Before vaccination, IFN-γ secretion in blood stimulated with *E. ruminantium* was not detectable (data not shown). After vaccination, although the magnitude of the response varied between animals, IFN-γ production was detected in vaccinated but not in control animals, despite similar IFN-γ responses to PMA + CI (Fig. 1). This was observed throughout the experiment (data not shown). Only low level of IFN-γ was produced in non-stimulated blood and in response to lysates from uninfected endothelial cells (Figs. 1

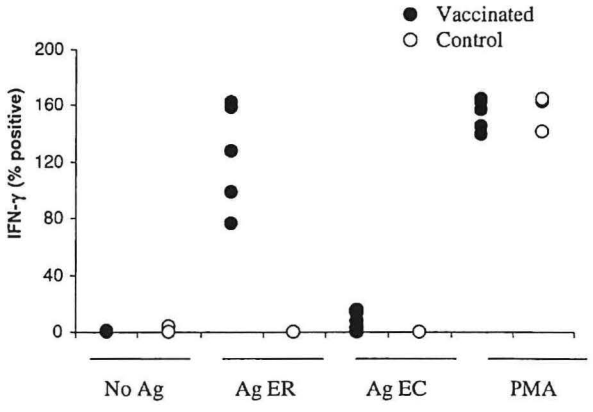

Fig. 1. IFN-γ secretion in blood collected from five *E. ruminantium* vaccinated (black markers) and three control (white markers) goats 3 weeks after the first injection, and incubated with: no antigens (No Ag), *E. ruminantium* antigens (Ag ER), uninfected endothelial cells (Ag EC) or PMA + CI (PMA). Results are expressed as percentages of the kit positive control and are means of duplicates.

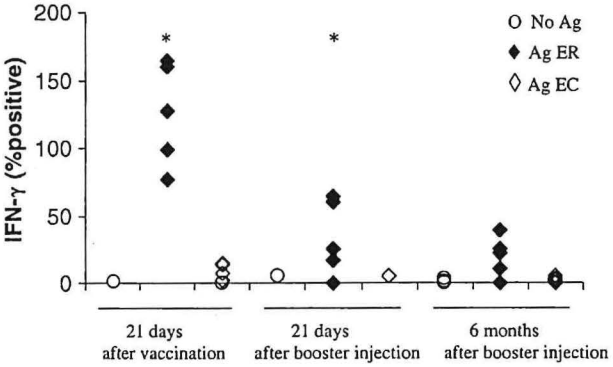

Fig. 2. Kinetics of IFN-γ secretion in five goats during vaccination with the killed *E. ruminantium* vaccine as measured by the whole blood assay. Blood was incubated with: no antigens (No Ag), *E. ruminantium* antigens (Ag ER) or uninfected endothelial cells (Ag EC). Results are expressed as percentages of the kit positive control and are means of duplicates. Asterisks indicate IFN-γ production induced by *E. ruminantium* antigens significantly above the negative controls (No Ag and Ag EC).

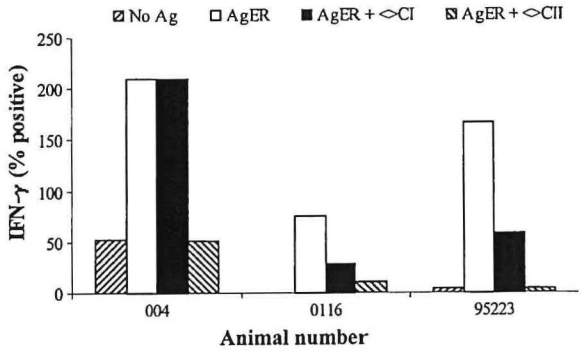

Fig. 3. Effect of anti-class I MHC (Ag ER + <>CI) and anti-class II MHC (Ag ER + <>CII) antibodies on *E. ruminantium*-induced (Ag ER) IFN-γ secretion in whole blood collected from vaccinated goats 1 week after the booster injection. Values from the non-stimulated control (No Ag) are also show and results are expressed as percentages of the kit positive control and are means of duplicates.

and 2). The highest IFN-γ production was generally observed in vaccinated animals 21 days after the first injection and there was a decreased of the recalled IFN-γ response to *E. ruminantium* antigens in most animals after the booster injection and after 6 months (Fig. 2). IFN-γ production in response to *E. ruminantium* Ag was significantly different ($P < 0.05$) from non-stimulated blood and non-infected endothelial cells after the first inoculation and 21 days after the booster injection, but not anymore after 6 months (Fig. 2).

3.2. Effect of anti-classes I and II MHC antibodies

In all animals tested, antibodies against class II MHC inhibited almost completely (>95%) the IFN-γ secretion induced by *E. ruminantium* (Fig. 3). Interestingly, in two out of three animals, more than 50% of *E. ruminantium*-induced IFN-γ secretion was also blocked when antibodies against class I MHC were added. Finally, antibodies were not toxic for the cells since they did not affect PMA-induced secretion of IFN-γ (data not shown).

3.3. Characterization of E. ruminantium-specific caprine T cell subsets that produce IFN-γ

We first used stimulated whole blood to mimic in vivo conditions as much as possible. However, low responses were generally observed most likely due to removal of activated cells by adherence to the tube during density gradient purification, which prompted the use of PBMC. Two-color flow cytometric analysis was performed to determine the phenotype of IFN-γ producing cells in *E. ruminantium*-stimulated PBMC. As shown in Table 1, IFN-γ+ cells were present within CD4+ and CD8+ populations of immune PBMC after stimulation with *E. ruminantium* antigens. The relative capacity of CD4 and CD8 to produce IFN-γ was comparable as shown by similar percentages but also by mean intensities of fluorescence (MIF, Table 1). This effect was specific of *E. ruminantium* stimulation because no IFN-γ was detected in the presence of uninfected endothelial cell antigens. Before the booster injection, we observed in all vaccinated animals tested (Table 1) that both CD8+ and CD4+ T cells subsets produced IFN-γ after *E. ruminantium* stimulation. In animals 95,208 and 95,223 some response was also observed in the WC1+ population but at a lower percentage and intensity of fluorescence. Two months after the booster injection a similar response was still detectable in two out of three animals. There was no detectable induction of IFN-γ in PBMC from control animals (Table 1). As shown in Fig. 4, the overall contribution of CD4+ and CD8+ T cells to the production of IFN-γ by immune PBMC was comparable, whereas WC1+ T cells played a negligible role.

Statistical analysis indicated significant differences ($P < 0.05$) between vaccinated and control animals

Table 1
Relative percentages of each T cell subsets (CD8+, CD4+ and WC1+ T cells) producing IFN-γ in vaccinated and control animals

Animals	T cell subsets[a]								
	CD8+ IFN-γ+			CD4+ IFN-γ+			WC1+ IFN-γ+		
	No Ag[b]	Ag ER[c]	Ag EC[d]	No Ag	Ag ER	Ag EC	No Ag	Ag ER	Ag EC
Vaccinated animals									
One month after the first inoculation									
95208	0.03	**5.11** *661*	ND[e]	0.11	**6.63** *535*	ND	0.05	**4.73** *394*	ND
95223	0.34	**6.58** *706*	ND	0.10	**7.13** *560*	ND	0.18	**2.89** *326*	ND
0045	0.55	**3.04** *312*	ND	0.47	**3.61** *294*	ND	0.37	0.07	ND
Cutoff[f]	1.09			0.86			0.68		
Two months after the second inoculation									
95208	2.16	**10.21**	2.86	1.07	**5.60**	1.46	0.49	**1.77**	0.52
95223	0.11	**7.00**	0.22	0.02	**6.54**	0.11	<0.01	0.48	<0.01
0045	0.08	0.01	<0.01	<0.01	0.25	<0.01	<0.01	<0.01	<0.01
Cutoff	4.36			2.20			1.01		
Control animals									
0029	0.25	0.36	0.26	0.05	0.09	0.09	<0.01	<0.01	<0.01
0046	0.05	0.27	ND	<0.01	<0.01	ND	<0.01	0.03	ND
0102	0.03	0.33	0.04	0.03	0.01	<0.01	<0.01	0.00	<0.01
Cutoff	0.47			0.10			<0.01		

[a] Percentages of CD4+, CD8+ or WC1+ T cell sub-populations that stained with mAb to intracellular IFN-γ above the isotype-matched control mAb. Boldface numbers indicate percentages above cutoff. Numbers in italic represent the mean intensity of fluorescence (MIF) of IFN-γ+ populations.

[b] Medium alone.

[c] *E. ruminantium* antigens.

[d] Non-infected endothelial cells antigens.

[e] Not done.

[f] Cutoff values were calculated as follow: mean percentage of IFN-γ positive cells in the absence of antigen plus 3 × S.D. (see also Section 2).

for CD4+ IFN-γ+ and CD8+ IFN-γ+ but not WC1+ IFN-γ+ T cells before the booster injection. Two months after the booster injection, differences were significant only for IFN-γ+ CD4+ T cells.

4. Discussion

The aim of the present study was to characterize the cells capable of producing IFN-γ in goat vaccinated with the killed *E. ruminantium* vaccine. Emphasis was put on the use of short incubation time and whole PBMC rather than enriched T cell lines, to minimize the bias introduced by the in vitro culture, and, because we believe it better corresponds to in vivo conditions. *E. ruminantium*-induced secretion

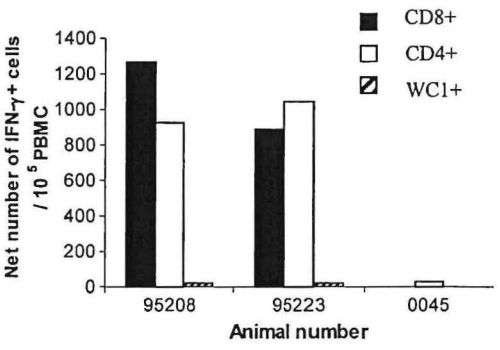

Fig. 4. Net contribution of different T cell subsets (CD8+, CD4+ and WC1+ T cells) to the production of IFN-γ by *E. ruminantium* restimulated immune PBMC collected 2 months after the booster injection. Results are expressed as the number of cells of a given subset per 10^5 PBMC that stained for IFN-γ in the presence of *E. ruminantium* antigens minus the value obtained in control medium cultures.

and production of cytoplasmic IFN-γ were measured by ELISA and by flow cytometry, respectively. The first method clearly shows that IFN-γ is produced in blood from vaccinated but not control animals upon in vitro restimulation with *E. ruminantium* antigens. After the booster injection, a decrease of IFN-γ response occurred, which suggest that antigen specific T cells that produce IFN-γ after an antigenic recall may leave the circulation and enter different compartments of the immune system. As we have previously observed (Esteves et al., 2002), the level of IFN-γ produced between animals varied greatly which may be explained by the high genetic diversity of Creole goat species. Subsequent analysis using flow cytometry indicated that immunization elicited CD4+ and CD8+ T cells that stained positive for cytoplasmic IFN-γ after antigenic recall in vitro. IFN-γ+ WC1+ cells were also observed but at much lower percentages or intensities of fluorescence. Moreover, because the percentage of circulating WC1+ T cells was much lower than CD8+ and CD4+ T cells, it is likely that their contribution to the overall IFN-γ production is less important. In fact, tricolor staining of CD4+, and CD8+ T cells and IFN-γ+ revealed that more than 90% of the IFN-γ+ population is comprised of CD4+ CD8− and CD4− CD8+ T cells in similar proportions (data not shown).

Surprisingly, the relative capacity of immune CD8+ T cells to produce IFN-γ was equal or superior to CD4+ T cells (Table 1 and Fig. 4). Vaccination with a killed vaccine generally induces a preponderant CD4 response due to the privileged presentation of exogenous antigens in association with class II MHC molecules. However, growing evidence suggest that exogenous antigens and killed pathogens can also be presented by class I MHC molecules (Szalay et al., 1995; Reimann and Schirmbeck, 1999; Wick and Ljunggren, 1999; Rhee et al., 2002). In our model, production of IFN-γ by CD8+ T cells was confirmed by blocking experiments. In two out of three animals, anti-class I MHC antibodies reduced by more than 50% the secretion of IFN-γ in *E. ruminantium*-stimulated blood. This experiment also indicates that the production of IFN-γ by CD8+ T cells is not simply due to a bystander effect. Moreover, CD4+ T cells were shown to play a key role since anti-class II MHC antibodies blocked more than 95% of the production in all animals tested. Thus, the *E. ruminantium* responding CD8+ T cells observed in this study are likely to be conventional CD8+ T cells that need the help of CD4+ T cells to become functional. Finally, taken together, results from blocking experiments and flow cytometry also suggest that main sources of IFN-γ in sensitized circulating PBMC are T lymphocytes, and not NK cells.

Our results are similar to what has been shown in *Mycobacterium bovis*-infected cattle, where both CD8+ and CD4+, and not WC1+ T cells produced IFN-γ after antigenic recall with soluble antigens (Walravens et al., 2002). There also, CD8+ T cells needed the help of CD4+ T cells in order to produce IFN-γ. In *M. tuberculosis*-infected mice, protection by CD8+ T cells is mediated by the production of IFN-γ rather than their cytotoxic activity (Tascon et al., 1998). We have recently shown in goats that in vitro stimulation with *E. ruminantium* antigens provoked the up regulation of interleukin-2 receptor expression on CD4+ and CD8+ T cells, but not WC1+ T cells (Totté et al., 2002). There is, however, no report, apart from the present study, on *E. ruminantium*-specific CD8+ effector T cells in ruminants. Nonetheless, studies in mice suggest that both CD4+ and CD8+ T cells can play a role in protection (Byrom et al., 2000; Du Plessis et al., 1991).

In conclusion, this study underlines the key role of CD4+ T cells in the production of IFN-γ by immune goat PBMC and describes, for the first time in ruminants, *E. ruminantium*-specific CD8+ effector T cells. Our results also highlight the importance in most vaccinated goats of CD8+ T cells as a source of this potent *E. ruminantium*-inhibitory cytokine. Thus, a recombinant vaccine will probably need to incorporate antigens containing both CD4+ and CD8+ T cells epitopes, in order to ensure a high level of protection against *E. ruminantium* infections.

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