Réponses immunitaires de la peau des souris BALB/c infestées par des nymphes de la tique *Ixodes ricinus*: infiltration cellulaire et production de cytokines

par

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Liste des abbréviations

ARNm: Acides ribonucléiques messagers
CD: Classe de Différenciation
CDD: Cellules Dendritiques Dermales
CL: Cellules de Langerhans
CPAg: Cellules de Présentation d'Antigène
DTH: Delayed-Type Hypersensitivity
ELAM: Endothelial-Leukocyte Adhesion Molecule
Ia: Antigènes d'histocompatibilité de classe II (souris)
ICAM: Intercellular Adhesion Molecule
LFA: Leukocyte Function-associated Antigen
PM: Poids moléculaire
VCAM: Vascular Cellular Adhesion Molecule
VLA: Very Late Antigen

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1. INTRODUCTION

1.1. Développement de la tique *Ixodes ricinus*

Les tiques sont des arthropodes hématophages, appartenant à l'Ordre des Acarina. Cet Ordre contient plusieurs Superfamilles, dont celle des Ixodoidea divisée en 3 Familles. La Famille des Ixodidae (ou tiques dures), la Famille des Argasidae (ou tiques molles) et la Famille des Nutellidae. *Ixodes ricinus* appartient à la Famille des Ixodidae, comprenant plus de 200 espèces (Hoogstraal et Aeschlimann, 1982).

Le cycle de développement des Ixodidae est fondamentalement identique pour toutes les espèces. De l'œuf, éclôt une larve hexapode qui va muer en nymphe après avoir pris un repas sanguin sur un hôte vertébré (oiseaux, petits mammifères, Homme). La nymphe se nourrit généralement sur les mêmes types d'hôtes que la larve, puis va muer en adulte mâle ou femelle. Les adultes doivent à leur tour se gorger sur un hôte (Ongulés, Canidés, homme), période pendant laquelle a lieu la copulation. Chez *I. ricinus*, la copulation est possible en dehors de l'hôte (Graf, 1978). Les tiques Ixodides ont des repas sanguins qui durent longtemps (4 à 7 jours), comparé aux Argasides qui mettent quelques heures pour se nourrir. A la fin du repas sanguin, la femelle se détache pour pondre des œufs, puis meurt. Les tiques mâles d'*I. ricinus* se nourrissent très peu.

1.2. Importance des tiques en tant que vecteurs de maladies

Aussi bien les Ixodides que les Argasides jouent un rôle économique et médical important par la transmission de nombreuses maladies au bétail et à l'Homme. Ainsi, des virus, des bactéries (*Borrelia*), des protozoaires et helminthes parasites peuvent être transmis par les tiques (Aeschlimann, 1991). Les pathogènes sont souvent injectés avec la salive pendant que l'ectoparasite est en train de se nourrir (Binnington et Kemp, 1980). La prise de sang est interrompue par de brèves périodes de salivation toutes les 5-30 secondes (Kaufman, 1989).

1.3. Développement d'une résistance de l'hôte contre les piqûres des tiques

De nombreux hôtes acquièrent une résistance après une ou plusieurs infestations avec des tiques. Le développement de l'ectoparasite en est affecté, ce qui se traduit par une diminution du pourcentage des tiques fixées, une baisse de

poids et du rendement de ponte des tiques nourries sur des animaux résistants (Brown, 1985; 1988; Allen, 1989; Brossard et al., 1991). Les mécanismes de cette réponse immune de l'hôte ont été étudiés le plus souvent avec des modèles expérimentaux. Il a été montré qu'aussi bien des composantes d'une réponse humorale que d'une réponse cellulaire, ainsi que l'activation du système complément, pouvaient participer à la protection, souvent partielle, de l'hôte contre les tiques.

1.3.1. Réponses humorales

Les bases immunologiques de l'immunité anti-tiques ont été partiellement établies par les travaux de Trager (1939). Cet auteur démontra que le transfert passif d'immun-sérum de cobayes résistants aux larves des tiques *Dermacentor variabilis* protégeait partiellement des animaux indemnes d'une infestation avec la même espèce. Le transfert d'immun-sérum de lapins résistants aux adultes d'*I. ricinus* confère aussi une protection partielle chez les receveurs (Brossard et Girardin, 1979). Les anticorps IgG anti-tiques participent également à la réponse immune contre les larves d'*A. americanum* chez les lapins et les cobayes (Brown et al., 1982b). Néanmoins, dans d'autres systèmes expérimentaux, les anticorps anti-tiques n'interviendraient pas dans l'acquisition de résistance. Ainsi, le transfert passif d'immun-sérum de cobayes résistants contre les larves d'*Ixodes holocyclus* et de *D. andersoni* ne confère aucune protection aux receveurs (Bagnall et Rothwell, 1974; Wikel et Allen, 1976a). Pourtant, le traitement des cobayes avec de la cyclophosphamide, à des doses appropriées qui inhibent la fonction des lymphocytes B, bloque l'acquisition de résistance contre les larves de *D. andersoni* (Wikel et Allen, 1976b).

1.3.2. Réponses immunes à médiation cellulaire

Le transfert passif de cellules immunes d'animaux résistants contre les tiques confère habituellement une meilleure immunité chez les receveurs qu'un transfert passif d'immun-sérum (Bagnall, 1975). Ainsi, une protection plus importante contre les larves de *D. andersoni* est obtenue après un transfert de cellules ganglionnaires qu'après celui de sérum de cobayes résistants (Wikel et Allen, 1976a). Les populations de cellules enrichies en lymphocytes T sont plus efficaces que celles enrichies en lymphocytes B. Par ailleurs, le traitement de lapins avec de la cyclosporin A abaisse la résistance aux tiques *I. ricinus* adultes (Girardin et Brossard, 1990). De plus, dans ce même système, la résistance est renforcée par un

traitement des lapins avec de l'IL-2 recombinante (Schorderet et Brossard, 1994). Ces travaux démontrent l'importance de la composante cellulaire dans l'immunité anti-tiques.

1.3.3. Le système complément

Le système complément participe aussi à l'acquisition de la résistance contre les tiques. La déplétion du C3 par traitement avec du facteur de venin de cobra chez des cobayes infestés par les larves de *D. andersoni* bloque son expression (Wikel et Allen, 1977). Ainsi, il a été noté une augmentation du nombre et du poids des tiques nourries sur les animaux traités. L'étude histologique du site de fixation des tiques sur ces animaux révèle un nombre réduit de basophiles, suggérant le rôle chimiotactique du complément envers ces cellules et leur importance dans l'élaboration d'une réponse protectrice. Chez les lapins infestés par *I. ricinus*, le taux sérique de C3 augmente et atteint un maximum en troisième infestation (Papatheodorou et Brossard, 1987). Le taux de C3 dans l'intestin moyen des ectoparasites s'élève aussi au cours des infestations. Aussi, l'hypothèse d'une activation du système complément au niveau de l'épithélium intestinal de la tique a-t-elle été émise, avec comme conséquence une perturbation de la physiologie de ce tissu (Brossard et Papatheodorou, 1990). Chez les cobayes résistants à *D. andersoni*, un dépôt d'antigènes salivaires, de composants du système complément et d'anticorps IgG a été visualisé par immunofluorescence à la jonction dermo-épidermale près du rostre des tiques (Allen et al., 1979).

1.4. L'interface peau-tique

La fixation et la nutrition de la tique sur l'hôte donnent lieu à une réponse locale faisant intervenir les composantes du système immunitaire de la peau. Une lésion de nutrition cutanée, nécessaire au repas sanguin, est formée.

1.4.1. Le système immunitaire de la peau

La peau a été longtemps considérée comme une barrière mécanique à diverses agressions du milieu extérieur. Durant ces dernières décennies, il est apparu qu'elle ne remplissait pas seulement cette fonction, mais qu'elle jouait aussi un rôle important dans l'initiation d'une réponse immunitaire primaire. Ainsi, Streilein (1978, 1983) définit le "Skin Associated Lymphoid Tissue" comme un ensemble de cellules comprenant les kératinocytes, les cellules de Langerhans, les lymphocytes T

résidents, les cellules endothéliales des vaisseaux sanguins. Ces dernières dirigent la migration des lymphocytes T dans le derme; les antigènes transportés par les CL sont présentés aux lymphocytes dans les ganglions lymphatiques régionaux. Bos et Kapsenberg (1986) ont par la suite introduit le terme "Skin Immune System" qui comporte les constituants cellulaires et humoraux participant aux défenses immunitaires de la peau.

Les kératinocytes, sentinelles de la peau

Les kératinocytes représentent plus du 90% des cellules épidermales. Par la synthèse de lipides et de diverses protéines de structure, dont les kératines, ils contribuent à la formation de la barrière cutanée (Elias et Brown, 1978). Ils interviennent aussi dans la réponse immunitaire de la peau. On leur attribue au moins deux fonctions. D'abord, celle de sentinelle par une réponse non spécifique à une perturbation de la barrière cutanée. Ils produisent un large éventail de cytokines, parmi lesquelles le TNF-α, l'IL-1α, l'IL-1β, l'IL-6 et l'IL-8 (Luger et Schwartz, 1990) en réponse à différents stimuli non spécifiques, comprenant les irradiations aux rayons ultraviolets, des agents chimiques irritants et des stimuli mécaniques. Le profil des cytokines varie qualitativement et quantitativement selon la nature du stimulus. Des facteurs chimiotactiques pour les neutrophiles et les lymphocytes T sont égalememt induits, de même que l'expression de molécules d'adhésion comme l'ICAM-1 (Dustin et al., 1988; Wawryk et al., 1989). La rétention des lymphocytes T dans la peau interviendrait par interaction entre l'ICAM-1 et son ligand, le LFA-1. D'autre part, les kératinocytes fonctionneraient comme CPAg car ils expriment, *in vitro*, des Ia après une incubation avec de l'IFN-γ (Nickoloff et Turka, 1993). Cette cytokine induit aussi l'expression d'ICAM-1 sur ces cellules. Ainsi, elles peuvent amplifier la réponse immunitaire, comme cela a été proposé dans certaines dermatoses telles que le psoriasis (Nickoloff et al., 1991).

Les lymphocytes T de la peau, un rôle dans la surveillance immune

Dans la peau humaine indemne, 2% des lymphocytes T cutanés sont présents dans l'épiderme, avec en majorité un phénotype $CD8^+$ (Bos et al., 1987). Les lymphocytes T restants sont regroupés autour des vénules post-capillaires du derme. Ces aggrégats contiennent approximativement le même nombre de lymphocytes T $CD4^+$ et $CD8^+$. Les lymphocytes B ne colonisent pas la peau indemne. Environ 16% des lymphocytes T du sang périphérique expriment une glycoprotéine de surface de 200 KDa, connue sous le nom de CLA (Cutaneous Lymphocyte-

associated Antigen) (Picker et al., 1990). En outre, plus de 85% des lymphocytes T infiltrant la peau dans différentes dermatoses expriment cet antigène. Le récepteur du CLA pourrait être une sélectine E, en l'occurrence l'ELAM-1 (Picker et al., 1991) qui représenterait une molécule d'adhésion pour des populations de T de la peau. Ces lymphocytes assurent la surveillance immune de ce tissu (Mackay, 1991).

Une nouvelle population de lymphocytes T dendritiques Thy 1^+ a été identifiée au niveau de l'épiderme des souris (Tschachler et al., 1983; Bergstresser et al., 1983). Il s'agit de lymphocytes T intraépithéliaux qui ne portent pas les marqueurs CD4 et CD8 et n'expriment pas les molécules Ia. De plus, les récepteurs des antigènes sont constitués par les chaînes polypeptidiques γ/δ, au lieu des chaînes α/β présents sur les lymphocytes T conventionnels. La fonction de ces cellules γ/δ dans le système immunitaire de la peau est encore mal connue. Néanmoins, elles montrent, *in vitro*, des propriétés fonctionnelles similaires aux lymphocytes T α/β (Allison et Havran, 1991).

Les cellules de Langerhans, les cellules dendritiques dermales et les macrophages: CPAg de la peau et des ganglions lymphatiques régionaux

Les CL ont été décrites pour la première fois par Langerhans (1868). Leur rôle dans la réponse immunitaire fut établi plus tard (Shelly et Juhlin, 1976). Les CL, cellules dendritiques de l'épiderme, sont caractérisées par la présence d'inclusions cytoplasmiques denses aux électrons, appelés granules de Birbeck (Birbeck et al., 1961). La présence de ces granules est associée avec la présentation des antigènes. Récemment en effet, il a été démontré *in vitro* que les CL étaient munies d'un pouvoir de phagocytose (Souza et al., 1993). Les cellules dendritiques mâtures ne peuvent plus digérer les antigènes en fragments peptidiques; cette incapacité s'accompagne d'une disparition des granules de Birbeck (Stössel et al., 1990). Les CL sont efficaces pour induire une réponse immunitaire primaire. Elles expriment constitutivement des antigènes Ia (Klareskog et al., 1977; Rowden et al., 1977). Chez l'homme, elles expriment aussi l'ICAM-1 et le LFA-3, qui sont des structures indispensables dans le processus de présentation des antigènes (Bierer et al., 1988; Dougherty et al. 1988). En outre, les CL produisent des cytokines dont l'IL-1 et l'IL-6 (Schreiber et al., 1992). Chez des cobayes résistants aux tiques, les CL digèrent et présentent les antigènes de ces ectoparasites aux lymphocytes T des ganglions de drainage (Nithiuthai et Allen, 1985). Le traitement des animaux aux rayons U.V. inhibe la fonction des CL (Cruz, 1992) et abolit l'expression de la résistance contre les tiques (Nithiuthai et Allen, 1984).

Le terme de cellules dendritiques introduit par Steinman et Cohn (1973), rend compte de la morphologie caractéristique de ces cellules *in vitro*; il rassemble une sous-population particulière de cellules capables de présenter les antigènes (Steinman, 1991; Bos et Kapsenberg, 1993). Les cellules dendritiques dérivent de cellules souches de la moelle osseuse qui donnent aussi naissance aux macrophages et aux granulocytes (Inaba et al., 1993). L'antigène capté par les CL au niveau de l'épiderme est transféré à travers les vaisseaux lymphatiques aux ganglions de drainage. Pendant cette migration, les CL changent de morphologie (cellules voilées) et deviennent des cellules interdigitées dans le paracortex. Ces dernières présentent l'antigène aux lymphocytes T. *In vitro*, les cellules dendritiques se comportent différemment selon leur état de maturation. Contrairement aux cellules fraîchement isolées, les cellules en culture pendant 3 jours sont incapables de digérer les proteines natives. Mais elles excèlent dans la présentation de peptides aux lymphocytes T et dans l'induction d'une prolifération primaire de ces cellules (Inaba et al., 1986; Romani et al., 1989; Cruz et Bergstresser, 1990; Steinman, 1991). D'autres CPAg, les macrophages par exemple, n'induisent pas efficacement une réponse immune primaire, mais plutôt une prolifération de lymphocytes T à mémoire (Steinman, 1991).

1.4.2. Les réactions cutanées aux piqûres de tiques

Les tiques, fixées dans la peau, se nourrissent sur une lésion de nutrition dont la formation implique, du moins en partie, la participation des neutrophiles (Tatchell et Moorhouse, 1970). Très souvent, des études histologiques ont été menées afin d'établir une corrélation entre la nature des cellules inflammatoires du site de fixation et l'acquisition de résistance par l'hôte. Les cobayes infestés avec des larves de *D. andersoni* acquièrent une résistance, et ils développent une hypersensibilité cutanée à basophiles (Allen, 1973). Le même type de réponse locale a aussi été observé chez des cobayes infestés avec d'autres espèces comme *I. holocyclus* (Bagnall, 1975), *A. americanum* (Brown et Askenase 1981). Le transfert à des animaux infestés par *A. americanum* d'anticorps anti-basophiles abolit l'expression de la résistance (Brown et al., 1982a). Les basophiles, soit environ 1% des leucocytes circulants chez le cobaye et le lapin, ne résident pas normalement dans la peau. Leur recrutement est la conséquence d'une réponse immunitaire locale faisant intervenir les lymphocytes T et des anticorps (Askenase, 1992). Chez des lapins infestés avec des adultes d'*I. ricinus*, une infiltration de mastocytes, de basophiles souvent en voie de dégranulation et d'éosinophiles a été observée (Brossard et Fivaz, 1982). Les basophiles circulants sont sensibilisés par des anticorps anti-tiques au

cours des infestations (Brossard et al., 1982). Les lapins infestés par cette espèce développent des réactions cutanées immédiate et une DTH, ce qui a été montré après une injection intradermale d'antigènes de glandes salivaires d'*I. ricinus* (Girardin et Brossard, 1985). L'infiltration fréquente de basophiles et de mastocytes en voie de dégranulation dans le site de fixation des tiques chez les lapins et les cobayes a conduit les chercheurs à définir le rôle de l'histamine dans l'acquisition de la résistance. Chez des bovins infestés par *Boophilus microplus* et des cobayes par *D. andersoni*, le taux d'histamine cutanée est corrélé avec le degré de résistance exprimé par l'hôte (Willadsen et al., 1979; Wikel, 1982). De plus, le traitement des animaux avec des antagonistes des récepteurs H1 et H2 ou avec de la mépyramine, un anti-histaminique H1, altère la résistance contre les tiques (Wikel, 1982; Brossard, 1982). Les médiateurs libérés lors de la dégranulation des mastocytes, notamment l'histamine, permettraient la migration des basophiles au site de fixation des tiques en augmentant la vasoperméabilité locale (Brown, 1985). La dégranulation ultérieure des basophiles entretiendrait la réponse inflammatoire locale et amplifierait l'infiltration des leucocytes. Le rejet d'*A. americanum* n'est pas altéré par un traitement de cobayes avec de la mépyramine (Brown, 1985). Il semblerait ainsi que l'histamine ne soit pas le seul médiateur impliqué dans l'acquisition de résistance contre cette espèce.

La réponse immune contre les stades immatures de tiques a aussi été étudiée dans des modèles murins. Des femelles de souris BALB/c acquièrent une résistance contre les larves de *D. variabilis* (Den Hollander et Allen, 1985a). Les tiques se nourrissent moins et en nombre plus faible. Mastocytes et éosinophiles, dont la plupart sont dégranulés, neutrophiles et cellules mononucléées infiltrent en grand nombre la peau infestée. Selon les auteurs, les mastocytes et les éosinophiles seraient indispensables à l'acquisition de résistance. Cette hypothèse paraît renforcée par l'utilisation de souches de souris W/W^v génétiquement déficientes en mastocytes. En effet, ces animaux restent susceptibles aux larves d'*Haemaphysalis longicornis* (Matsuda et al., 1985). L'intervention des mastocytes dans l'immunité anti-tiques ne serait pas généralisée à toutes les espèces de tiques. En effet, les souris W/W^v acquièrent une résistance contre les larves de *D. variabilis* (den Hollander et Allen, 1985b). Dans ce cas, l'infiltration de basophiles et d'éosinophiles près du rostre des tiques chez les souris W/W^v résistantes compenserait l'absence de protection conférée par les mastocytes (Steeves et Allen, 1990, 1991).

Les mécanismes complexes intervenant dans la réponse immune contre les tiques sont encore mal compris. Dans notre travail, nous compléterons les connaissances actuelles de la réponse inflammatoire et immunitaire des souris contre les piqûres de tiques.

1.5. Les cytokines, molécules clés dans la régulation de la réponse immunitaire

1.5.1. Définition et exemples

Le terme de cytokine a été introduit pour définir toute substance soluble, produite par les cellules lymphoïdes et non-lymphoïdes, exerçant des effets spécifiques sur des cellules cibles. Ce terme englobe les lymphokines, substances produites par les cellules lymphoïdes, et les monokines produites par les cellules de la lignée monocyte/macrophage. Afin d'exclure de cette définition des médiateurs telles que les hormones et d'autres petites molécules biologiquement actives (histamine, prostaglandines, leukotriènes, etc...), le terme de cytokine a été restreint aux médiateurs d'origine animale ayant un PM supérieur ou égal à 5 KDa. Ils exercent une action spécifique autocrine et /ou paracrine via des récepteurs (Meager, 1990). En général, ces molécules contrôlent les fonctions cellulaires qui assurent l'homéostase et les mécanismes de défense. La production de certaines cytokines n'a pourtant pas toujours un effet bénéfique sur la physiologie de l'hôte.

Les cytokines suivantes ont été considérées dans ce travail:

Interleukine-1 (IL-1)

L'interleukine-1 est une cytokine produite par un grand nombre de cellules comprenant les monocytes, les macrophages, les lymphocytes, les cellules endothéliales, les cellules dendritiques, les kératinocytes, les neutrophiles et les fibroblastes (Oppenheim et al., 1986). Initialement, l'IL-1 ou "Lymphocyte Activating Factor" a été définie comme étant une molécule, produite par les monocytes, qui augmente la réponse des lymphocytes T aux mitogènes et aux antigènes (Gery et al., 1972).

Deux formes d'IL-1 sont connues: l'IL-1α de PM de 22 KDa, et l'IL-1β de PM de 17 KDa qui est la forme prédominante (Lomedico et al., 1984; Auron et al., 1984). L'IL-1 associée à la membrane, serait la forme impliquée dans la présentation des antigènes aux lymphocytes T (Unanue et al., 1987).

L'injection d'IL-1 à des lapins mobilise les neutrophiles de la moelle osseuse vers le sang périphérique puis dans les sites enflammés (Warren, 1990). Cette cytokine agirait indirectement en provoquant la production d'IL-8 qui est elle chimiotactique pour les neutrophiles. Elle provoque aussi fièvre et production de prostaglandines de la série E2 (Durum et al., 1985). Elle induit l'expression des ELAM-1 et augmente celle des ICAM-1 sur la paroi des vaisseaux sanguins, ce qui

facilite l'extravasation des leucocytes dans le site enflammé (Warren, 1990). L'IL-1 stimule la prolifération des fibroblastes et induit la production de collagène, ce qui lui confère un rôle important dans le phénomène de cicatrisation (Luger et Schwartz, 1990). Elle augmente aussi la production de l'IL-2 et l'expression des récepteurs de cette cytokine. Elle stimule la différenciation et la prolifération des lymphocytes B en les rendant plus sensibles aux effets des IL-4, IL-5 et IL-6 (Yamashita, 1987).

Tumor Necrosis Factor (TNF)

L'injection de LPS chez des souris s'accompagne de la nécrose de certaines tumeurs. Cette observation conduisit à la découverte du TNF (Old, 1985). Le TNF-α, de PM de 17 KDa, montre une importante homologie de séquence (près de 80%) chez la souris, le lapin et l'homme (Beutler et Cerami, 1989). La principale source de TNF-α est constituée par les cellules de la lignée monocyte/macrophage. De nombreux autres types cellulaires produisent aussi cette cytokine, comme les lymphocytes, les mastocytes, les leucocytes polynucléaires, les astrocytes, les cellules dendritiques de la peau et les kératinocytes (Vassalli, 1992). Toutes les cellules nucléées répondent au TNF-α par l'intermédiaire de récepteurs spécifiques dont l'expression est augmentée par les cytokines IFN-γ et IL-2 (Tsujimoto et al., 1986; Thoma et al., 1990). Deux types de récepteurs, TNF-R1 de 55 KDa et TNF-R2 de 75 KDa, ont été identifiés aussi bien chez l'homme que chez la souris (Brockhaus et al., 1990; Lewis et al., 1991). Ils déterminent des réponses cellulaires distinctes (Tartaglia et al., 1991). Le TNF-R2 induit la prolifération des thymocytes et des lymphocytes T cytotoxiques *in vitro*, tandis que le récepteur TNF-R1 contrôle la cytotoxité des lymphocytes (Tartaglia et al., 1991). Le TNF-α exerce aussi un effet sur les vaisseaux sanguins en promouvant l'expression de molécules d'adhésion ELAM-1 et ICAM-1 (Zhang et al., 1992; Grau et Lou, 1993). L'adhésion des neutrophiles sur la paroi des vaisseaux en est augmentée. Il est impliqué indirectement dans la migration des neutrophiles et des lymphocytes vers la peau des patients souffrant du psoriasis, par exemple, en induisant la production d'IL-8, une cytokine chimiotactique pour ces types cellulaires (Barker et al., 1991; Nickoloff et al., 1991).

Le TNF-α participe à la réponse immunitaire contre les parasites. Ainsi, il augmente *in vitro* la toxicité des éosinophiles envers les schistosomules (Silberstein et David, 1986). La progression de la malaria cérébrale chez des souris est accompagnée d'une forte production de TNF-α (Grau et al., 1989). La neutralisation de cette cytokine avec des anticorps prévient l'issue fatale de la maladie chez ces animaux (Grau et

al., 1987). Le TNF-α joue sans doute aussi un rôle important dans le développement de la lèpre (Parida et Grau, 1993). Il participe aussi à la formation d'un granulome caractéristique chez les souris infectées par *Mycobacterium bovis* (Kindler et al., 1989). Un traitement avec des anticorps anti-TNF prévient la formation de cette lésion et empêche l'élimination de la bactérie.

Interféron gamma (IFN-γ)

L'IFN-γ a été découvert par son activité antivirale (Wheelock, 1965). L'IFN-γ murin contient 133 acides aminés (PM de 15.5 KDa) avec une homologie de séquence d'environ 40% avec l'IFN-γ humain (Gray et Goeddel, 1982). La source majeure de cette cytokine est constituée par les lymphocytes T activés. Des lymphocytes T $CD4^+$, les TH1, et tous les T $CD8^+$ produisent cette cytokine (Vilcek et al., 1985). Les "Natural Killer" la synthétisent également (Handa et al., 1983). Les effets de l'IFN-γ sur le système immunitaire sont multiples (Schreiber et Celada, 1985). Cette cytokine contrôle la sécrétion des différents isotypes d'anticorps par les plasmocytes (Snapper et Paul, 1987; Finkelman et al., 1988; Finkelman et al., 1990). Ainsi, elle inhibe fortement *in vitro* la production d'anticorps de classe IgE, IgG1, IgG2b et IgG3 chez la souris, tout en stimulant la production des anticorps IgG2a. Elle bloque sélectivement la différenciation des lymphocytes B en plasmocytes produisant des IgM (Abed et al., 1994). D'autre part, *in vivo*, une suppression de la réponse humorale a été constatée lorsque l'IFN-γ est injectée pendant la phase initiale de la réponse immunitaire (Finkelman et al., 1988). Cette cytokine augmente l'expression des Ia et des antigènes de classe I sur les macrophages (Allen et Unanue, 1987). Elle représente la seule cytokine connue qui induit l'expression des molécules Ia sur des cellules qui en sont normalement dépourvues (Farrar et Schreiber, 1993). Son influence sur la présentation des antigènes ne réside pas seulement dans l'augmentation de l'expression des Ia, mais aussi dans l'élévation du taux d'enzymes intracellulaires, indispensables à la dégradation des antigènes (Allen et Unanue, 1987). L'IFN-γ stimule aussi l'expression des ICAM-1 sur les macrophages, ce qui permet leur interaction avec les lymphocytes T (Pober et al., 1986; Mantovani et Dejana, 1989). Elle augmente aussi l'expression des récepteurs de haute affinité pour le Fc des IgG sur les cellules de la lignée monocyte/macrophage (Erbe et al., 1990). Les phénomènes d'opsonisation en sont renforcés, ainsi que l'élimination de microorganismes. En outre, l'IFN-γ augmente le pouvoir de phagocytose et la cytotoxicité des macrophages par la production intense de métabolites toxiques (Farrar et Schreiber, 1993). La biosynthèse des composants C2, C4 et du facteur B par les macrophages

et les fibroblastes est aussi augmentée par l'IFN-γ (Strunk et al., 1985). Cette cytokine contrôle aussi l'expression des récepteurs du complément sur les phagocytes. Ces différents effets confèrent à cette cytokine un rôle très important dans l'élimination de microorganismes intracellulaires tels que les leishmanies.

Dans la peau, les cellules épidermales (cellules dendritiques Thy-1) produisent de l'IFN-γ (Matsue et al., 1993). Celui-ci induit l'expression des ICAM-1 sur les kératinocytes, ce qui expliquerait la rétention des lymphocytes T infiltrant l'épiderme dans le cas du psoriasis (Nickoloff, 1990; Nickoloff et al., 1991).

Interleukine-2 (IL-2)

L'IL-2, de PM de 15.5 KDa chez la souris, est une lymphokine principalement produite par les lymphocytes T auxilliaires. Elle possède 60% d'homologie de séquence avec l'IL-2 humaine (Morgan et al., 1976; Smith, 1980). L'IL-2 est une molécule centrale de la réponse immunitaire, qui est essentielle à la prolifération et la différenciation des lymphocytes T et B exposés à des antigènes *in vitro* (Cantrell et Smith, 1984). *In vivo*, elle ne serait pas indispensable, car les thymocytes de souris génétiquement déficientes en cette cytokine prolifèrent et acquièrent leur maturité immunologique (Schorle et al., 1991; Kündig et al., 1993). L'IL-2 contribue au développement des lymphocytes T cytotoxiques, ce qui lui confère un rôle important dans l'immunité contre les virus (Mac Donald et Nabholz, 1986). L'injection de la proteine recombinante induit l'expression du gène codant pour le TNF-α chez des souris (Remick et al., 1991).

L'IL-2 recombinante a été utilisée pour traiter le mélanome malin chez l'homme (Dummer et al., 1991). Le traitement provoque une infiltration de lymphocytes T $CD4^+$ et une augmentation de l'expression des ICAM-1 sur les cellules endothéliales vasculaires, probablement à la suite d'une production d'IFN-γ par les lymphocytes activés. Chez des lépreux, l'injection intradermale, pendant 8 jours de 10 μg par jour d'IL-2, provoque une accumulation de cellules mononucléées au site d'injection, notamment de lymphocytes T $CD4^+$ (Kaplan et al., 1991). Le traitement ralentit la progression de la maladie par une augmentation de la réponse cellulaire locale.

Interleukine-4 (IL-4)

L'IL-4 de souris (PM de 20KDa) possède 50% d'homologie de séquence avec l'IL-4 humaine. Elle est principalement produite par les lymphocytes T activés (Howard et al., 1982; Paul et Ohara, 1987). D'autres types cellulaires (mastocytes et

basophiles) synthétisent aussi cette cytokine (Brown et al., 1987). Chez la souris, l'IL-4 assiste les lymphocytes B dans la production d'anticorps IgE et IgG1 tout en inhibant la production des IgM, IgG3, IgG2a et IgG2b (Coffman et al., 1986; Finkelman et al., 1986; Snapper et Paul, 1987). En outre, cette cytokine augmente l'expression des récepteurs de faible affinité des IgE sur les lymphocytes B (Hudak et al., 1987). Elle intervient dans les phénomènes d'allergie en provoquant la dégranulation des mastocytes et des basophiles (Capron et al., 1986). Elle promouvoit la croissance et la différenciation des lymphocytes B, mais aussi des mastocytes en synergie avec d'autres cytokines comme l'IL-3 (Noelle et al., 1984; Lee et al., 1986). Par ailleurs, elle augmente l'expression des molécules Ia sur les lymphocytes B et les macrophages (O'Garra et al., 1988). Cette cytokine favorise, *in vitro*, le développement des lymphocytes T $CD4^+$ en cellules effectrices produisant de l'IL-4, IL-5 et IL-10 en présence d'antigènes ou de mitogènes (Swain, 1993). En outre, il a été montré *in vitro* que l'IL-10 inhibe la production d'IFN-γ et d'IL-2 par les lymphocytes T.

L'IL-4 participe à l'élimination de cellules tumorales de la peau en favorisant l'infiltration et l'activation des éosinophiles (Tepper et al., 1989; Tepper, 1993). Cet effet semble indépendant de l'IL-5, car un traitement simultané avec de l'IL-4 et des anticorps anti-IL-5 ne bloque pas l'infiltration de ces cellules.

L'IL-4 induit l'expression des VCAM-1 sur les cellules endothéliales des vaisseaux, alors que l'expression des ICAM-1 et ELAM-1 n'est que partiellement inhibée par cette cytokine *in vitro* (Thornhill et Haskard, 1990; Thornhill et al., 1991; Schleimer et al., 1992). L'expression sélective des VCAM-1 par l'IL-4 sur les cellules endothéliales favorise l'infiltration des éosinophiles au détriment des neutrophiles dans des sites riches en IL-4. En effet, les éosinophiles expriment les récepteurs VLA-4 des VCAM-1, contrairement aux neutrophiles (Schleimer et al., 1992).

1.5.2. Profil de cytokines associé aux parasitoses

Chez la souris, des clones de lymphocytes T $CD4^+$ ont été sudivisés en sous-populations selon le profil des cytokines produites (Mosmann et al., 1986). Les TH1 synthétisent principalement de l'IFN-γ et de l'IL-2, tandis que les TH2 produisent de l'IL-4, IL-5, et IL-10. Ces deux sous-populations contrôlent des réponses immunitaires différentes qui s'excluent mutuellement. Ainsi, les TH1 interviennent dans les réponses cellulaires (DTH et activation des macrophages); les TH2 assistent la réponse humorale (Mosmann et Coffman, 1989). Différents profils de cytokines ont été définis chez des souris infectées par des parasites intracellulaires

ou des helminthes (Sher et Coffman, 1992). Un des exemples les plus édifiants à propos de la dualité fonctionnelle des lymphocytes T CD4$^+$ en relation avec l'état immun de l'hôte concerne la leishmaniose (Liew, 1989). L'infection à *Leishmania major* occasionne une lésion cutanée qui guérit spontanément chez les souris C57BL/6. Par contre, chez les BALB/c, l'infection progresse, avec dissémination du parasite et mort des souris. La résistance ou la susceptibilité à ce parasite dépend du développement d'une réponse TH1 ou TH2. Les lymphocytes T CD4$^+$ de souris susceptibles contiennent des quantités élevées d'ARNm pour l'IL-4 et peu pour l'IFN-γ, contrairement aux lymphocytes T CD4$^+$ d'animaux résistants où la situation est inversée (Heinzel et al., 1989; 1991). La stimulation antigénique *in vitro* des lymphocytes d'animaux infectés aboutit à la production préférentielle d'IFN-γ ou d'IL-4 selon les souches de souris (Sadick et al., 1986). Le traitement des souris BALB/c avec des anticorps anti-CD4$^+$ les convertit en souche résistante par diminution de la réponse TH2 (Titus et al., 1985; Heinzel et al., 1989). Le même résultat est obtenu en traitant les souris susceptibles avec des anticorps anti-IL-4. La guérison de la maladie chez les souris résistantes s'accompagne d'une forte DTH cutanée et d'un titre d'anticorps très bas (Liew, 1989). La susceptibilité à l'infection est corrélée à une forte réponse en IgE des souris contre les antigènes parasitaires, alors que les réactions de DTH cutanée sont très faibles (Heinzel et al., 1989, Liew, 1989). L'élimination des parasites résulte de l'activation des cellules par l'IFN-γ avec la formation d'intermédiaires toxiques dans les macrophages (Murray et al., 1983; Sadick et al., 1986).

La même dichotomie du profil des cytokines a été observée chez des patients atteints de la lèpre (Yamamura et al., 1991). Cette maladie est causée par une bactérie intracellulaire, *Mycobacterium leprae*, à l'origine de la formation de lésions cutanées. Deux formes cliniques, tuberculinique et lépromateuse, ont été décrites. Dans la forme tuberculinique, la croissance du pathogène est inhibée; alors que dans la forme lépromateuse, les lésions cutanées nombreuses sont la conséquence d'une multiplication incontrôlée de la bactérie (Sieling et Modlin, 1992). La détection *in situ* des cytokines ou de leurs ARNm par immunohistochimie ou hybridation démontre un nombre plus élevé de cellules positives pour l'IFN-γ, l'IL-1α, l'IL-2 et le TNF-α dans la forme tuberculinique que dans la forme lépromateuse (Cooper et al., 1989; Arnoldi et al., 1990; Yamamura et al., 1991; Walker et al., 1992). Dans cette dernière, la présence d'IL-4, d'IL-5 et d'IL-10 est beaucoup plus marquée. Chez des souris infectées par la bactérie, une forte DTH cutanée est constatée dans la forme tuberculinique, alors qu'elle est inexistante dans la forme lépromateuse (Sieling et Modlin, 1992). Les lymphocytes T CD4$^+$ cutanés prédominent dans la forme tuberculinique, avec un rapport CD4$^+$: CD8$^+$ de 1.9:1 (Modlin et al., 1983).

L'expression des ICAM-1 par les kératinocytes est intense dans les cas d'une forte DTH, et l'épiderme contient aussi de nombreux lymphocytes LFA-1 positifs (Sieling et Modlin, 1992). Une situation contraire est observée dans la forme lépromateuse.

Peu d'études concernent les ectoparasites, notamment sur le profil de cytokines produites en relation avec la résistance ou la susceptibilité des hôtes. Une inhibition partielle de la production de cytokines par les macrophages péritonéaux (IL-1 et TNF-α) et par les lymphocytes T (IL-2 et IFN-γ) de souris a été observée *in vitro* en présence d'antigènes de glandes salivaires des tiques *D. andersoni* (Ramachandra et Wikel, 1992). Un traitement de lapins avec de l'IL-2 recombinante augmente la résistance contre les tiques *I. ricinus* (Schorderet et Brossard, 1994). Cet effet serait dû à une réponse cellulaire augmentée. Dans le même modèle, l'effet du TNF-α sur le développement des tiques a été estimé après injection de cette cytokine (Schorderet, 1993). Le TNF-α semble favoriser la nutrition des ectoparasites par une intensification des réactions inflammatoires locales.

Dans notre travail, la dynamique des cellules inflammatoires infiltrant le site de fixation des nymphes d'*I. ricinus* a été étudiée au cours de trois infestations de souris. La détection locale des cytokines IL-1α, TNF-α, IFN-γ, IL-2 et IL-4 par immunohistochimie et hybridation *in situ* a été entreprise dans le but de mieux caractériser le profil des cytokines produites localement lors d'infestations par les tiques.

2. BUTS DU TRAVAIL

Les buts de ce travail sont les suivants:

1. Etudier l'impact d'infestations répétées de souris BALB/c et de souris génétiquement déficientes en IL-4 par des nymphes d'*I. ricinus* sur le développement de ces ectoparasites.

2. Définir la réponse inflammatoire locale à la piqûre des tiques en identifiant et comparant les différents types cellulaires infiltrant leur site de fixation au cours de trois infestations successives.

3. Par immunohistochimie, évaluer quantitativement les populations de lymphocytes T $CD4^+$ et $CD8^+$ infiltrant le site d'attachement des tiques au cours d'infestations répétées; l'expression des molécules Ia et ICAM-1 jouant un rôle clé dans la dynamique de la réponse locale sera démontrée par la même technique.

4. Par immunohistochimie et hybridation *in situ*, établir le profil des cytokines produites (IL-1α, TNF-α, IFN-γ, IL-2 et IL-4) localement et dans les ganglions lymphatiques de drainage, en marquant les protéines sécrétées et/ou les ARN messagers.

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ABSENCE OF ACQUIRED RESISTANCE TO NYMPHAL *IXODES RICINUS* TICKS IN BALB/C MICE DEVELOPING CUTANEOUS REACTIONS

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ABSTRACT: BALB/c mice underwent 3 successive infestations with 15 *Ixodes ricinus* nymphs. No resistance was acquired as assessed by evaluating tick attachment, duration of blood meal, weights of engorged nymphs, and molting success. However, the hosts developed cutaneous immediate- and delayed-type hypersensitivity reactions when reinfested. Histological examination of tick attachment sites showed that inflammatory cells consisting of neutrophils, eosinophils, and mononuclear cells (lymphocytes and monocytes) infiltrated the skin more intensively during reinfestations. The number of intact mast cells did not vary between successive infestations, whereas the number of degranulated mast cells increased in the early stages of reinfestations. Basophils, which represent 12% of total infiltrating cells, were only observed and quantified in the skin of reinfested mice using transmission electron microscopy (TEM). Degranulating eosinophils were also observed by use of TEM.

BALB/c mice acquired resistance after repeated infestations with *Dermacentor variabilis* larvae (Den Hollander and Allen, 1985a). Reduction in numbers and in weights of fed larvae were observed. Prominent infiltration of mast cells and eosinophils in the skin seemed to correlate with the acquisition of resistance. Moreover, according to Matsuda et al. (1985) mast cell-deficient mice (W/W^v) failed to acquire resistance after repeated infestations with larval *Haemaphysalis longicornis*. Moreover, these mice acquired resistance after successive infestations with *D. variabilis* larvae (Den Hollander and Allen, 1985b). Basophils, which sometimes have been reported to be absent or rare in mice (Askenase, 1977; Galli, 1987), were observed in resistant mast cell-deficient mice (Steeves and Allen, 1990). They have been associated with murine resistance against ticks.

In guinea pigs, acquired resistance to *Dermacentor andersoni* larvae was associated with cutaneous basophil hypersensitivity (Allen, 1973).

Rabbits expressed resistance after single infestation with *Ixodes ricinus* adults (Bowessidjaou et al., 1977). More degranulated mast cells and basophils were observed at the ticks' attachment sites on reinfested hosts (Brossard and Fivaz, 1982). With a degranulation test, basophils were shown to be sensitized to salivary gland antigens (Brossard et al., 1982). In addition, skin of resistant rabbits was sensitized (immediate and delayed type) to these antigens (Girardin and Brossard, 1985). Cutaneous hypersensitivities were reduced after a treatment with cyclosporin A (Girardin and Brossard, 1989), and acquired resistance was inhibited (Girardin and Brossard, 1990).

Until now, few immunological and histopathological studies have been reported for mice undergoing infestations with ixodid nymphal ticks (Riek, 1959; Saito and O'Hara, 1961). In the present paper, we report a study about skin sensitivity (immediate and delayed type) to tick antigens in BALB/c mice undergoing 3 successive infestations with *I. ricinus* nymphs. The development of these nymphs was studied for the 3 infestations. A sequential quantitative analysis of inflammatory cells near the tick rostrum is also presented. Numbers of intact or degranulated mast cells, eosinophils, neutrophils, and mononuclear cells (monocytes and lymphocytes) are compared during and between the 3 infestations. Using transmission electron microscopy (TEM), we confirm our observations revealing and quantifying basophils in the skin of reinfested mice.

MATERIALS AND METHODS

Ticks (*I. ricinus*) were reared in our laboratory as previously described (Graf, 1978). Female BALB/c mice were obtained from BRL (Basel, Switzerland). They were 8–12 wk old at the time of infestations. Mice were anesthetized with pentobarbiturate Vetanarcol (Veterinaria, Zürich, Switzerland), 2.5% in distilled water injected (intramuscular) at 10 μl/g body weight. Hairs on the flanks were clipped and plastic capsules (18 mm in diameter) were glued to the skin using a mixture of 4 parts colophonium and 1 part beeswax as previously described (Nelson and Kozub, 1980). A collar was placed around the neck of each mouse to prevent grooming. Three successive infestations were done with 15 nymphs per mouse. The duration of each infestation was between 4 and 7 days. No skin site was infested more than once. Mice were given 7 days free of ticks

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TABLE I. Biology of *Ixodes ricinus* nymphs for 3 infestations. No statistical differences are noted.

Infestation (n = 15 each)	Tick fixation (%)	Duration of blood meal (days, $\bar{x} \pm$ SD)		Weight of engorged tick (mg, $\bar{x} \pm$ SD)		Tick molting (%)
		♀ Nymphs	♂ Nymphs	♀ Nymphs	♂ Nymphs	
1	79	5.75 ± 0.76*	5.00 ± 0.85†	5.00 ± 0.93*	2.87 ± 0.37†	93
2	78	5.56 ± 0.78‡	5.00 ± 0.82§	4.81 ± 0.61‡	2.91 ± 0.30§	91
3	76	5.60 ± 0.70‖	5.14 ± 0.66#	4.82 ± 0.70‖	2.88 ± 0.42#	90

* n = 64.
† n = 101.
‡ n = 78.
§ n = 83.
‖ n = 81
n = 74.

between successive infestations. Weights of engorged nymphs were recorded on the drop-off day. Nymphs were kept individually in tubes placed at 28 C with approximately 100% relative humidity, until the molt (Graf, 1978) to separate males and females. To study the influence of repeated host infestations on the development of *I. ricinus* nymphs, the following parameters were used: number of attached ticks, duration of the blood meal, weights of male and female engorged nymphs, and molting success.

Approximately 150 unfed *I. ricinus* nymphs were homogenized in 1 ml of 50 mM phosphate buffer, 120 mM NaCl, pH 7.4, containing 1 mM proteinase inhibitor phenylmethylsulfonyl fluoride and 10 mM ethylene diaminetetraacetic acid. The homogenate was centrifuged at 10,000 *g* for 10 min, and the supernatant portion was dialyzed overnight at 4 C in 25 mM phosphate buffer, 120 mM NaCl, pH 7.4. Protein concentration was determined by the Coomassie protein assay (micromethod; Pierce, Pully, Switzerland), using bovine serum albumin as standard.

Twenty days after the beginning of primary (group 1), secondary (group 2), or tertiary infestation (group 3), 50 μl of a nymphal extract (1 mg/ml) or 25 mM phosphate buffer, 120 mM NaCl, pH 7.4, was injected into the footpads in each infestation group of mice. Skin thickness was measured with a micrometer each 10 min during the first hour and 6, 24, 48, and 72 hr thereafter.

Histological study was done during the 3 successive infestations. Two skin biopsies per mouse, including attached ticks, were taken from 3 mice 12, 24, 72, or 96 hr after the ticks were placed on their hosts. Some mice prepared to be infested but without ticks served as control. Skin biopsies were fixed immediately for 24 hr at 4 C in 4% formalin in 10 mM phosphate buffer, 150 mM NaCl, pH 7.4, washed 3 times in buffer, dehydrated to 95% ethanol, infiltrated, and embedded in glycol methacrylate (JB-4 embedding medium, Polysciences, München, Germany). Sections (1.5 μm thick) perpendicular to the skin's surface were cut through the tick attachment sites and stained with toluidine blue or Giemsa solution (Merck, Basel, Switzerland) (Böck, 1984). Two sections were viewed from each mouse. At each tick feeding site a 1.24-mm^2 area comprising the total area infiltrated by inflammatory cells was examined. Mast cells, cosinophils, neutrophils, and mononuclear cells (lymphocytes and monocytes) were counted using an Olympus Vanox-S microscope.

For TEM, 2 skin biopsies per mouse (3 mice per infestation) were obtained from each infestation group 72 hr posttick attachment and fixed for 2 hr in 2.5% glutaraldehyde at room temperature in 0.1 M cacodylate buffer (pH 7.2) with 3% sucrose, then washed 3 times and postfixed for 1 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through a graded series of acetone, infiltrated, and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections (60–80 nm thick) were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM201 TEM. To quantify basophils under the TEM, copper grids with an alphabetical mark were used (Balzers, Fürstentum, Liechtenstein). The surface of each section was determined by estimating the surface of 1 square on a micrograph.

A Mann–Whitney *U*-test was used to compare durations of blood meal and weights of engorged nymphs between infestations. The same test was also used to analyze the evolution of inflammatory cell infiltrates during and between the 3 infestations. An evaluation of the percentages of tick attachment and engorged nymphs was done with Fisher's exact test. A Dunnett's test was used to analyze data from the cutaneous hypersensitivity test.

RESULTS

The mean percentage of tick attachment did not significantly vary during the 3 infestations (Table I). The mean duration of the blood meal and weight of engorged male and female nymphs did not differ between the 3 infestations. Also, the percentage of engorged nymphs that molted did not vary.

A significant increase of skin thickness was measured 10 min after tick antigen injection in mice after 2 and 3 infestations ($P < 0.001$; Fig. 1). This immediate-type reaction was still detected 20 min after injection ($P < 0.001$).

A slight delayed-type reaction, 24 hr after the injection of tick antigen, was visible after the primary and tertiary infestation. After the secondary infestation, this delayed skin reaction was more pronounced ($P < 0.05$).

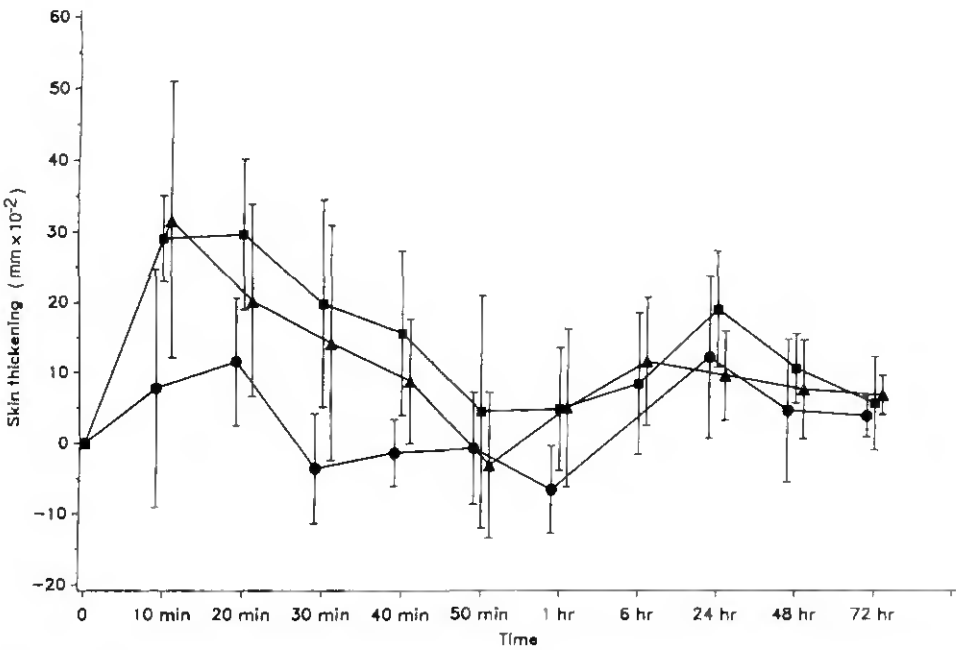


FIGURE 1. Evolution of the skin thickening of BALB/c mice infested with *Ixodes ricinus* nymphs. Thickening of footpad that received phosphate-buffered saline has been subtracted from the thickness of the footpad that received tick antigen. Infestation 1, circles; infestation 2, squares; infestation 3, triangles. Means ± SD are shown.

Histological examination of skin biopsies taken from control mice (without tick infestation) showed no neutrophil and eosinophil infiltration. Mast cells as well as some mononuclear cells were observed.

The tick's rostrum penetrated deeply into the dermis (Fig. 2). Twelve hours after nymphs were placed on mice both the epidermis and the dermis looked normal.

Intact mast cells and degranulated mast cells did not vary in number during this infestation (Fig. 3). The number of infiltrating eosinophils remained very low at all times (Fig. 3). A relatively low number of neutrophils had infiltrated the dermis 24 hr after tick attachment. It was significantly increased 72 hr and 96 hr after initiation of this infestation ($P < 0.01$, Fig. 3). Mononuclear cells were detected in the dermis 24 hr posttick attachment and did not differ after 72 hr. However, they had infiltrated the dermis in higher numbers at 96 hr postattachment ($P < 0.05$, Fig. 3).

In secondary infestations, the epidermis looked normal; however, a few inflammatory cells, including neutrophils and lymphocytes, occasionally were seen. There was an obvious cellular infiltration in the dermis and the hypodermis. The blood vessels appeared dilated and were filled with inflammatory cells. There was no variation in number of intact mast cells; nevertheless, degranulating mast cells were more numerous at 12 hr, then decreased 24 hr after tick attachment ($P < 0.01$, Fig. 3). Eosinophils were already de-

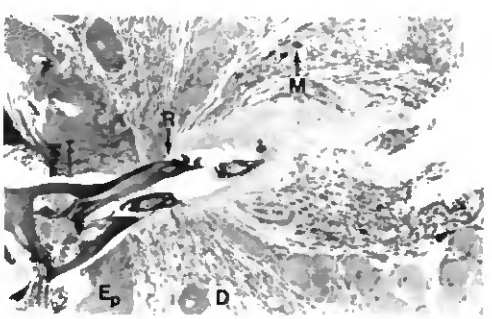


FIGURE 2. Skin biopsy of BALB/c mouse infested with *Ixodes ricinus* nymphs. Tertiary infestation, 72 hr. Note the degranulating mast cells (M) near the rostrum (R) in the dermis (D). Ep, epidermis.

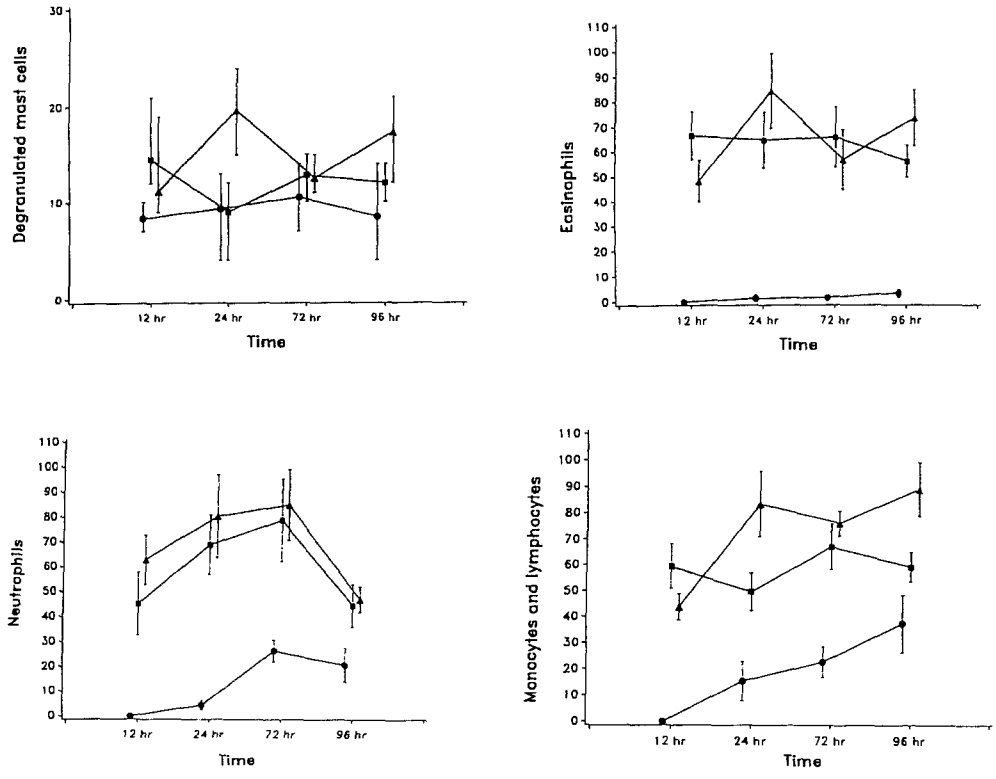


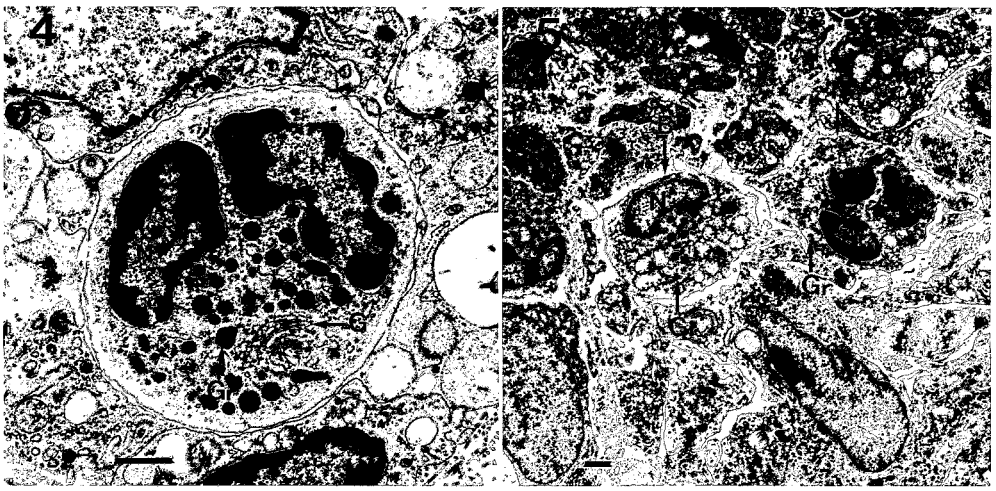
FIGURE 3. Evolution of inflammatory cells during and between infestations. Infestation 1, circles; infestation 2, squares; infestation 3, triangles. Results are represented by joining the mean numbers of cells at each time. Means ± SD are shown.

tected in the dermis after 12 hr, and their number did not change during this infestation (Fig. 3). There was an evident infiltration of neutrophils after 12 hr and they had increased in number 72 hr posttick attachment ($P < 0.05$, Fig. 3), then decreased at the end of the infestation ($P < 0.05$). Mononuclear cells infiltrated the skin in constant numbers until 24 hr after the beginning of this infestation, then increased after 72 hr ($P < 0.05$) and remained stable until the end of the infestation (Fig. 3).

In tertiary infestations, again no variation in number of intact mast cells was observed. Nevertheless, the number of degranulated mast cells varied. Thus, it reached a first peak after 24 hr ($P < 0.05$) and a second one after 96 hr ($P < 0.05$, Fig. 3). The number of eosinophils often fluctuated during this infestation (Fig. 3). It reached its highest value 24 hr posttick attachment ($P < 0.01$). Neutrophils infiltrated the dermis, the hypodermis, and occasionally the epidermis. Until 72 hr their number slightly increased and then decreased significantly after 96 hr ($P < 0.01$). At that time, they infiltrated in lower numbers than 12 hr posttick attachment (Fig. 3). Mononuclear cells infiltrated preferentially the dermis and the hypodermis, but a few were seen occasionally in the epidermis. Their numbers increased from 12 to 24 hr ($P < 0.01$) and reached their highest level 96 hr posttick attachment ($P < 0.05$, Fig. 3).

The number of intact mast cells did not differ among the 3 infestations, but degranulated mast cells usually were more numerous during the reinfestations (Fig. 3). Thus, it increased at an early stage (12 hr) of the secondary infestation when compared to the primary infestation ($P < 0.01$). It also increased 24 hr after the tertiary infestation compared to the previous ones ($P < 0.01$).

Compared to the primary infestation, eosinophils infiltrated the dermis 4–7 times more



FIGURES 4, 5. Electron photomicrographs of the skin of BALB/c mice infested with *Ixodes ricinus* nymphs. **4.** Extravascular basophil, tertiary infestation, 72 hr. Note the differences in electron density of the granules. G, Golgi; Gr, granules; N, nucleus. Bar = 1 μm. **5.** Extravascular basophil and neutrophil, secondary infestation, 72 hr. Note the differences between basophil and neutrophil granules. B, basophil; Gr, granules; Ne, neutrophils; N, nucleus. Bar = 1 μm.

abundantly at reinfestations ($P < 0.01$, Fig. 3). They infiltrated the skin in smaller numbers at the early stage of tick attachment in the tertiary infestation compared to the secondary infestation ($P < 0.05$). On the contrary, they were more numerous after 24 and 96 hr during the tertiary infestation than in the secondary infestation ($P < 0.05$ and $P < 0.01$).

Neutrophils infiltrated the skin 3–4 times more abundantly at reinfestations ($P < 0.01$, Fig. 3). Between the secondary and the tertiary infestation, they had a similar quantitative evolution, except at 12 hr posttick attachment when they infiltrated the skin in higher numbers in the tertiary infestation ($P < 0.05$).

Like neutrophils, mononuclear cells infiltrated the skin 3–5 times more at reinfestation ($P < 0.01$, Fig. 3). There was a more pronounced infiltration 24 hr and 96 hr posttick attachment during the tertiary infestation ($P < 0.01$).

Basophils were identified by TEM according to the criteria of Dvorak et al. (1982). Both extravascular and intravascular cells were observed in the skin of reinfested mice. They had a lobated nucleus as well as bigger cytoplasmic granules that were rounder and occurred in smaller numbers than those found in neutrophils (Figs. 4, 5). Electron densities often varied in basophil granules (Fig. 4). The Golgi apparatus, cytoplasmic vesicles, and multivesicular bodies sometimes were seen.

At the primary infestation, basophils were very rare or absent. The mean number of basophils counted 72 hr posttick attachment by TEM in secondary infestations was $31.8 \pm 11.4/1.27$ mm^2 and $28.8 \pm 10.2/1.27$ mm^2 in tertiary infestations. This represented approximately 12% of the inflammatory cells infiltrating the skin at that time, with 24% being eosinophils, 33% neutrophils, and 31% mononuclear cells. No significant variation in the number of infiltrated basophils was observed between the secondary and the tertiary infestation 3 days after tick attachment.

Degranulated eosinophils were observed at tick attachment sites by use of TEM.

DISCUSSION

These experiments demonstrate that BALB/c mice undergoing repeated infestations with *I. ricinus* nymphs fail to acquire resistance. Tick attachment, duration of the blood meal, weights of engorged nymphs, and the success of molting do not differ among infestations.

BALB/c mice develop a cutaneous immediate- and a slight delayed-type cutaneous hypersensitivity after reinfestations. Histological examination of their skin showed a slight cellular response during primary infestation that became

more pronounced at reinfestations. The number of intact mast cells did not differ significantly among the 3 infestations, whereas number of degranulated mast cells increased at the early stage of reinfestations (12 hr). These observations correspond to the development of skin sensitivity to tick antigens (immediate type) in reinfested mice. Degranulation of some mast cells during primary infestation may be due to mechanical injury. Esterases in the saliva can also hydrolyze cholesterol esters in mast cell membranes (Geczy et al., 1971). Cutaneous hypersensitivities were associated with the expression of tick resistance in guinea pigs and rabbits (Allen, 1973; Girardin and Brossard, 1985). Histamine liberated by degranulated mast cells and basophils was shown to participate in the acquisition of tick resistance (Brossard, 1982; Wikel, 1982).

The importance of eosinophils and basophils in tick resistance was emphasized in several systems. Thus, resistant BALB/c mice infested with *D. variabilis* larvae showed a prominent infiltration of eosinophils at tick feeding sites (Den Hollander and Allen, 1985b). In cattle, lysosomal enzymes released by degranulating eosinophils are thought to participate in the formation of epidermal vesicles often involved in tick rejection by resistant hosts (Schleger et al., 1976). Our results showed a marked infiltration of eosinophils in the skin of reinfested mice. Free eosinophilic granules were also observed by use of TEM, suggesting that some degranulation occurred. The presence of "reverse granules" confirms this, as their occurrence was proposed to represent a preliminary sign of degranulation (McLaren et al., 1983).

Brossard et al. (1982) demonstrated that circulating basophils in rabbits infested with *I. ricinus* adults were progressively sensitized against tick salivary gland antigens. More degranulated basophils were also observed at the larval *Rhipicephalus appendiculatus* feeding sites in actively sensitized guinea pigs (McLaren et al., 1983). Using TEM, Steeves and Allen (1990) showed the presence of basophils in the skin of resistant mast cell-deficient mice (W/W^v) infested with *D. variabilis* larvae. We also observed basophils by use of TEM in the dermis of reinfested mice, where they infiltrated in an appreciable number (12%). We noted the presence of basophil granules that were less electron dense. These were proposed to represent immature forms rather than degranulating cells (Dvorak et al., 1982). However, it is not excluded that some degranulation of basophils occurred because mast cells were observed to degranulate in the same section.

Our results showed that murine hosts can fail to acquire nymphal tick resistance despite the presence of basophils, degranulating mast cells, and eosinophils at tick feeding sites. Moreover, skin of infested mice became sensitized (immediate and delayed types) against tick antigens. The slight delayed-type hypersensitivity observed could be due to local release of lymphokines by mononuclear cells.

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PUBLICATION II

Immunology 1994 **82** 596–602

Infiltration of $CD4^+$ $CD8^+$ T cells, and expression of ICAM-1, Ia antigens, IL-1α and TNF-α in the skin lesion of BALB/c mice undergoing repeated infestations with nymphal *Ixodes ricinus* ticks

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SUMMARY

The skin cellular immune response of BALB/c mice was examined during three successive infestations with nymphal *Ixodes ricinus* ticks. An immunohistochemical analysis of skin cryostat sections 72 hr post-tick attachment revealed that $CD4^+$ T cells outnumbered $CD8^+$ T cells in all infestations. The $CD4^+$: $CD8^+$ T-cell ratio was 2·2 : 1 in the primary infestation, then increased to 3·2 : 1 and 4·7 : 1 in the secondary and tertiary infestations. No B lymphocytes (CD45R) were detected in the skin of control and infested mice. A positive staining of intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells, dendritic cells and some other mononuclear cells was observed in the dermis. Also, a strong positive staining of Ia antigens on dendritic cells and infiltrated mononuclear cells was noted. The staining pattern was more intense and positive cells increased in number in the skin of re-infested mice compared to the primary infestation. In addition, cells such as epidermal keratinocytes, dermal dendritic cells and infiltrated mononuclear cells positive for the 'pro-inflammatory' cytokines interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) were localized in the skin of infested mice, as detected at the mRNA level by *in situ* hybridization and at protein level by immunostaining with antibodies. These results suggest that an antigen was presented to infiltrating T lymphocytes which then became activated. This event may explain the cutaneous delayed-type hypersensitivity previously described in tick-infested BALB/c mice. Importantly, this cutaneous reaction was not sufficient to protect the mouse against tick re-infestation. Furthermore, ICAM-1 could mediate, at least in part, the extravasation of inflammatory cells into the skin of infested mice.

INTRODUCTION

Several studies have been reported about the inflammatory response in hosts infested with ticks.[1,2] BALB/c mice repeatedly infested with larval *Dermacentor andersoni* acquired resistance and their skin showed an infiltration of mast cells, neutrophils, eosinophils and mononuclear cells.[1] It was suggested that mast cells and eosinophils may participate in the acquisition of resistance to ticks in BALB/c mice. On the other hand, a local activation of complement has been suggested in resistant guinea-pigs infested with *D. andersoni* larvae.[3] Tick antigens injected into the skin via the saliva during feeding were shown to be trapped by Langerhans' cells, and presented to T lymphocytes in the draining lymph nodes.[4] BALB/c mice infested with nymphal *Ixodes ricinus* ticks failed to acquire resistance, despite the appearance of a marked cellular infiltrate in the skin, including neutrophils, eosinophils, basophils and mononuclear cells (monocytes and lymphocytes).[5] In addition, an increase in number of locally degranulating mast cells was observed.[5] The skin of infested mice became sensitized to tick antigens (immediate and delayed-type). Little importance was given to the infiltrating mononuclear cells in the skin of murine hosts infested with ticks. Therefore, attempts were made to characterize lymphocyte phenotypes infiltrating the skin of infested BALB/c mice using antibodies to mouse cell-surface markers CD4, CD8 and CD45R (mouse B lymphocytes).

Intercellular adhesion molecule-1 (ICAM-1) can mediate, at least in part, the adhesion of leucocytes to the vessel wall, and extravasation.[6] Its expression has been shown to be up-regulated by cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α),[7] which possess many other biological properties including the regulation of the pathological process in the host response to various stimuli. For example, biologically active membrane-associated IL-1 may be the form which participates in activating lymphocytes.[8] Due to the lack of information about the local immune response to ticks, we also undertook this work to examine the local production of IL-1α and TNF-α and the local expression of Ia antigens and ICAM-1 in BALB/c mice infested successively with nymphal *I. ricinus* ticks.

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MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Iffa-Credo (Arbresle, France). They were between 8 and 12 weeks old at the time of infestation.

Ticks

Ixodes ricinus nymphal ticks, originally from the pathogen-free colony of our laboratory, were reared as described previously.[9]

Infestations

Fifteen *I. ricinus* nymphs (approximately 6 weeks old) per mouse were placed within a plastic capsule glued to the skin, as previously described.[5] Three successive infestations were done and no skin site was infested more than once. Mice were given 7 days free of ticks between successive infestations. Mice prepared to be infested, but without ticks, served as controls.

Tissue preparation

Skin biopsies, including attached ticks, were taken 72 hr post-tick attachment and embedded in Optimal Cutting Temperature compound (OCT; Bayer-Pharma, Zürich, Switzerland), then frozen in isopentane chilled in liquid nitrogen and stored at −70°. Cryostat sections (5 μm thick) were placed on poly-L-lysine-coated slides[10] and allowed to dry in air. Sections were fixed in acetone for 10 min at 4°, air-dried and hydrated in Tris-buffered saline (100 mM Tris–HCl, 150 mM NaCl, pH 7·6) for 2 min before immunostaining.

Antibodies

The following monoclonal rat IgG antibodies were used: anti-L3T4/CD4 (IgG2a), anti-Ia^d (IgG2a) (Boehringer-Mannheim, Rotkreuz, Switzerland), anti-CD8-biotin (IgG2a) (Gibco, Basel, Switzerland), anti-CD45R (IgG2a) for murine B cells (Pharmingen, Lugano, Switzerland) and anti-ICAM-1 (IgG2a) (British Bio-technology Products, Abingdon, U.K.).

Polyclonal rabbit anti-mouse IL-1-α and TNF-α were purchased from Genzyme (Boston, MA).

Immunohistochemistry

For anti-L3T4/CD4, CD45R, Ia^d and ICAM-1 antibodies, an indirect immunoperoxidase technique was used. After inhibiting the endogenous peroxidase activity with 0·1 M sodium azide and 0·04% hydrogen peroxide,[11] sections were incubated for 30 min at room temperature (21°) with primary antibodies diluted 1/100 in 100 mM Tris–HCl, 150 mM NaCl (TBS), pH 7·6, containing 0·2% bovine serum albumin (BSA; Sigma, St Louis, MO). Control slides received the diluent alone. After three washes (5 min each) in TBS, slides were incubated for 30 min at room temperature (21°) with peroxidase-conjugated sheep anti-rat Ig (Boehringer-Mannheim), diluted 1/200 in TBS–0·2% BSA.

A biotin–streptavidin immunoperoxidase technique (Histomark kit; KPL, Gaithersburg, MD) was used for anti-CD8 biotin rat monoclonal antibodies.

Peroxidase activity was demonstrated by incubation for 5 min in diaminobenzidine/tetrahydrochloride (DAB), containing hydrogen peroxide using the KPL peroxidase detection kit.[12] Sections were then counterstained with Mayer's haematoxylin solution (Fluka, Buchs, Switzerland) and mounted with fluoromount-G (SBA, Birmingham, AL).

A biotin–streptavidin–alkaline phosphatase complex (Histomark kit; KPL) was used for the detection of IL-1-α and TNF-α. Sections were blocked with normal goat serum 20 min before immunostaining. Slides were sequentially incubated with anti-IL-1-α and anti-TNF-α, respectively diluted 1/150 and 1/400 in TBS–0·2% BSA, biotinylated goat anti-rabbit IgG and streptavidin–alkaline phosphatase. All incubations were performed for 30 min at room temperature (21°). Several washes (5 min) in TBS were done between all incubations. Phosphatase activity was revealed with fast-red development. Sections were counterstained and mounted as described above. The omission of the primary antibodies and the development of alkaline phosphatase alone yielded a negative signal.

In situ *hybridization*

Biopsy specimens were frozen as described above and the *in situ* hybridization technique was performed.[13] The ultra-pure water used for cleaning slides and for preparation of hybridization solutions was treated in 0·1% diethylpyrocarbonate (DEPC; Sigma) and allowed to stand at room temperature overnight before autoclaving, in order to remove any contaminating RNases.[14]

Probes

The cDNA for murine IL-1-α and TNF-α were synthetic oligonucleotides (30 and 28 bases, respectively) complementary to murine mRNAs cytokines. Probes were labelled at the 5′ end with digoxigenin and were purchased from British Biotechnology Products.

Hybridization

Five-micrometre thick cryostat sections were fixed in 4% paraformaldehyde for 20 min at 4°. After several washes in 0·1 M phosphate-buffered saline, pH 7·2, free amino groups were acetylated by treatment with 0·25% acetic anhydride in 0·1 M triethanolamine, pH 8. Sections were prehybridized in hybridization buffer, consisting of 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7), 30% deionized formamide (Gibco), 1 × Denhardt's solution, 125 μg/ml sheared denaturated salmon sperm DNA (Gibco), for 1 hr at 37°. Slides were then drained and sections covered with 50 μl of hybridization buffer containing DNA probe at a concentration of 0·5 mg/ml. Sections were covered with siliconized coverslips, sealed with rubber cement and hybridized overnight at 37°.

Post-hybridization washing was performed in decreasing concentration of SSC (4 × –2 × –0·2 × SSC) with 30% formamide at 37°. After 15 min washing at room temperature in Tris-buffered saline (0·05 M Tris–HCl, 0·15 M NaCl, 2 mM $MgCl_2$, 0·1% BSA), pH 7·6, containing 0·1% Triton X-100, sections were incubated with sheep anti-digoxigenin–alkaline phosphatase conjugate (British Biotechnology Products) diluted 1/600 in TBS. Alkaline phosphatase activity was demonstrated using the β-chloroindolyl-phosphate–nitroblue tetrazolium (BCIP–NBT) medium using the digoxigenin detection kit (British Biotechnology Products) for 3–6 hr. Slides were then counterstained with Mayer's haematoxylin solution (Fluka).

Table 1. $CD4^+$ and $CD8^+$ T cells 72 hr post-tick attachment in 1·24 mm^2 of skin of BALB/c mice infested *I. ricinus* nymphs

	T lymphocytes		
	$CD4^+$ T cells	$CD8^+$ T cells	CD4/CD8 ratio
Normal skin	4·0 ± 0·0	3·66 ± 0·01	1·09
Infestation			
1	19·16 ± 1·01[†]	8·66 ± 1·20	2·2
2	78·33 ± 3·44(†) [†]	24·66 ± 3·97(*)	3·2
3	86·00 ± 12·16(†) [†]	18·33 ± 2·67(*)	4·7

( ) Comparison between re-infestation and primary infestation for each T-cell subpopulation.

[ ] Comparison between $CD4^+$ and $CD8^+$ T cells in each infestation.

*$P < 0·05$.

†$P < 0·01$.

Means ± SE are shown.

The cell counting was carried out around the tick rostrum.

Sections incubated with RNase A (Boehringer Mannheim) (0·1 mg/ml in 2 × SSC, 10 mM $MgCl_2$) for 1 hr at 37° and the application of the hybridization solution, without probe, served as negative control. This yielded the expected negative signal.

Microscopic evaluation

Two sections per mouse, for a total of three mice per infestation with nymphs, were viewed with an Olympus Vanox-S microscope and cells counted according to phenotypic distribution. A total area of 1·24 mm^2 was examined.

Statistical analysis

A non-parametric test Mann–Whitney *U*-test was performed to analyse the data from lymphocyte counting.

RESULTS

Normal skin

In non-infested mice, dendritic cells in the epidermis (Langerhans' cells), dermal dendritic cells, and resident macrophages stained positive for Ia antigens. ICAM-1 expression was confined to vascular endothelium and to some basal epidermal keratinocytes. No CD45R B cells were present, while a few $CD4^+$ and $CD8^+$ T cells were detected in the intact dermis. Immunostaining with anti-CD45R antibodies of axillary BALB/c mouse draining lymph node cryostat sections was performed as a positive control (data not shown). Some epidermal keratinocytes stained positive for both mRNA and secreted proteins IL-1α and TNF-α (data not shown).

Tick-infested skin

In the primary infestation, $CD4^+$ T cells outnumbered $CD8^+$ T cells, with a CD4/CD8 ratio of 2·2 (Table 1). T cells preferentially infiltrated the dermis and were rarely observed in the epidermis. No CD45R B lymphocytes were detected in the skin. Epidermal Langerhans' cells, dermal dendritic cells and a few infiltrating mononuclear cells stained positive for Ia antigens, while keratinocytes remained negative. ICAM-1 staining was confined to basal epidermal keratinocytes and dermal dendritic cells. An intense staining was observed on mononuclear cells in the dermis. Epidermal keratinocytes,

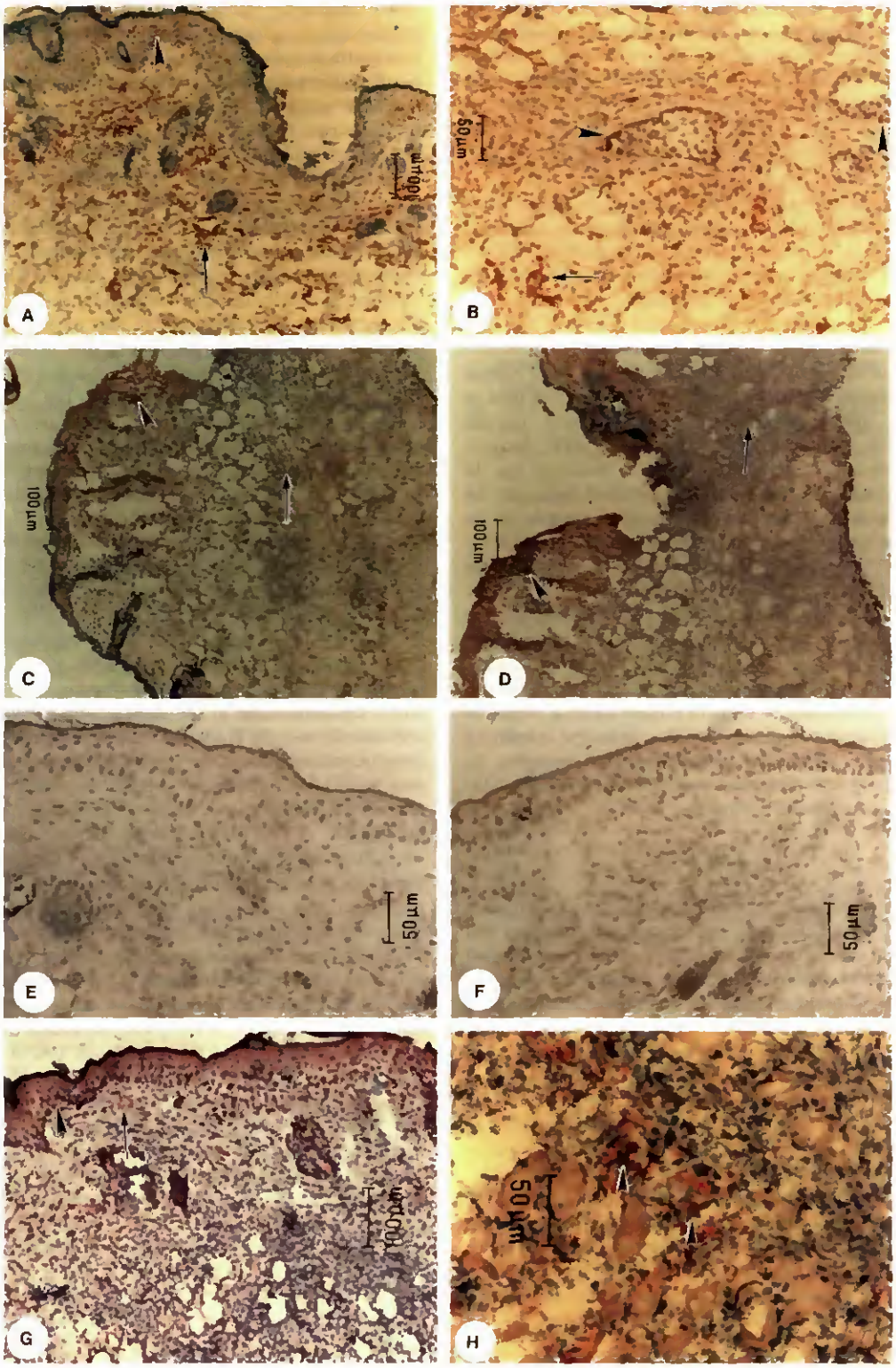
Table 2. Summary of results obtained by immunostaining with antibodies or using *in situ* hybridization on skin biopsies taken 72 hr post-tick attachment on BALB/c mice

	Immunostaining				*In situ* hybridization	
	Ia	ICAM-1	IL-1α	TNF-α	IL-1α	TNF-α
Control mice	+*, LC, DC, MC	±, VE, KC	±, KC	±, KC	±, KC	±, KC
Infestation 1	+, LC, DC, MC	±, KC, VE, MC, DC	+, KC, DC	+, KC, DC	+, KC, MC	+, KC, MC
Infestation 2	++, LC, DC, MC	++, KC, VE, MC, DC	+, KC, DC	++, KC, DC, MC	+, KC, MC	++, KC, MC
Infestation 3	++, LC, DC, MC	++, KC, VE, MC, DC	+, KC, DC	++, KC, DC, MC	+, KC, MC	++, KC, MC

*Intensity of coloured end product : ±, weak; +, light; ++, strong.

LC, Langerhans' cells; DC, dermal dendritic cells; KC, keratinocytes; MC, mononuclear cells; VE, vascular endothelial cells.

Figure 1. (*opposite*) (a) Expression of Ia antigens on Langerhans' cells in the epidermis (arrowhead), dendritic cells and infiltrated mononuclear cells (arrow) in the dermis of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. (b) Expression of ICAM-1 in the skin of BALB/c mice infested with *I. ricinus* ticks; secondary infestation, 72 hr. Note the positive staining on vascular endothelium (arrowheads) and some infiltrated mononuclear cells in the dermis (arrow). (c) Keratinocytes (arrowhead), primarily near the tick rostrum, and some infiltrating mononuclear cells (arrow) positive for IL-1α mRNA; tertiary infestation, 72 hr. (d) Keratinocytes (arrowhead) and some infiltrating mononuclear cells (arrow) positive for TNF-α mRNA in skin biopsy of BALB/c mice infested with nymphal *I. ricinus* ticks; tertiary infestation, 72 hr. (e) and (f) Control sections treated with RNase, and hybridized with IL-1α (e) and TNF-α (f) probes. No localizing signal. (g) IL-1α in the skin of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. Epidermal keratinocytes (arrowhead), hair follicles and some dendritic cells (arrow) appear positive. (h) TNF-α in the skin of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. Note the positivity of some infiltrating mononuclear cells in the dermis (arrowheads).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primarily near the tick rostrum, showed a positive signal for IL-1α and TNF-α mRNA. Also, a few infiltrating mononuclear cells showed detectable amounts of IL-1α and TNF-α mRNA. At the protein level, TNF-α was detected on dermal dendritic cells and in the same cell types that were positive for this cytokine's mRNA, contrasting with IL-1α, whose protein was only detected in epidermal keratinocytes and dermal dendritic cells.

In the secondary and tertiary infestations, $CD4^+$ and $CD8^+$ T cells infiltrated the skin in higher numbers compared to the primary infestation ($P < 0{\cdot}01$, $P < 0{\cdot}04$). $CD4^+$ outnumbered $CD8^+$ T cells in re-infestation ($P < 0{\cdot}003$), with a CD4/CD8 ratio of 3·2 in the secondary infestation and 4·7 in the tertiary infestation (Table 1). Again, no CD45R B lymphocytes were detected in re-infested mice.

Staining of Ia and ICAM-1 antigens became more intense with re-infestation and many more cells were positive than in the primary infestation (Fig. 1a, b, respectively). Dermal dendritic cells and infiltrated mononuclear cells (macrophages, monocytes) showed a positive staining for Ia antigens, in addition to some Langerhans' cells in the epidermis. Epidermal keratinocytes remained negative for Ia antigens, but some basal keratinocytes stained positive for ICAM-1. The intensity of ICAM-1 staining on keratinocytes remained at constitutive levels observed in normal skin.

Mononuclear cells infiltrating the dermis showing positive signals for IL-1α and TNF-α mRNA (Fig. 1c, d, respectively) became more numerous than in primary infestation. The staining pattern in the epidermis was identical to that obtained in the primary infestation. The IL-1α protein staining pattern in re-infested mice was similar to the primary infestation (Fig. 1g). TNF-α protein was detected on keratinocytes, on dermal dendritic cells and some mononuclear cells (Fig. 1h) (a summary of the results is shown in Table 2).

DISCUSSION

BALB/c mice undergoing repeated infestations with nymphal *I. ricinus* ticks fail to acquire resistance.[5] Inflammatory cell infiltration into the dermis of nymphal *I. ricinus* tick-infested BALB/c mice was shown to peak 72 hr post-tick attachment.[5] The epidermis showed a slight infiltration of inflammatory cells.[5] During the three successive infestations, 72 hr post-tick attachment, $CD4^+$ T cells outnumbered $CD8^+$ T cells, while no B cells were detected in the skin of susceptible BALB/c mice. Differences in number and phenotypes of lymphocytes subpopulations recruited in the skin have been suggested to determine the outcome of challenge with metacyclic trypanosomes.[15] A marked infiltration of $CD4^+$ T-helper cells, $T19^+$ ($CD4^-$, $CD8^-$) T cells and $CD45R^+$ (B cells) was shown in the skin of sheep infected with the larvae of *Lucilia cuprina* during both primary and secondary infestations compared with control sites.[16] The presence of B cells in the skin could represent an important event because it may allow a protective humoral response to be effected at the level of the skin, as previously suggested.[15]

The selective infiltration of T cells in the skin of nymphal *I. ricinus*-infested BALB/c mice may be partially mediated by adhesion molecules such as ICAM-1. Our results showed that ICAM-1 is expressed on vascular endothelium and the mononuclear cells infiltrating the skin of re-infested mice. Newly infiltrating mononuclear cells also stained strongly positive for Ia antigens. The involvement of Ia antigens in T-cell activation has been demonstrated.[17,18] In addition, a low T-cell proliferative response to antigen occurred when expression of Ia on antigen-presenting cells was reduced.[19]

In murine contact allergic dermatitis, the expression of ICAM-1 on epidermal keratinocytes precedes infiltration of the epidermis by T lymphocytes.[20] The adhesion of eosinophils to endothelial cells appeared to be favoured by the expression of adhesion molecules such as ICAM-1. Anti-ICAM-1 antibodies inhibit eosinophilia of the bronchial mucosa.[21] Mast cell degranulation also participates, *in vitro*, in the rapid and transient induction of adhesion molecules for leucocytes on nearby venular endothelium.[22,23] This event depends on the local release of TNF.[22] A previous study has demonstrated an increase of degranulated mast cells in the skin of BALB/c mice infested with nymphal *I. ricinus* ticks.[5] The co-expression of ICAM-1 and Ia antigens could facilitate adhesion and subsequent MHC class II antigen-restricted antigen presentation to T lymphocytes, as previously suggested.[24]

Both IL-1α and TNF-α were detected at mRNA and protein levels at the tick fixation site of BALB/c mice. The detection of mRNA and the secreted proteins IL-1α and TNF-α in the epidermis of non-infested mice is in accordance with previous studies showing the presence of these cytokines in unstimulated mouse epidermal cells.[25,26] IL-1α and TNF-α locally produced in the skin of infested mice may up-regulate ICAM-1 expression on the endothelial cell.[7] TNF-α injected intracutaneously was shown to increase binding of anti-ICAM-1 monoclonal antibody in the skin of *Papio anubis*.[27] TNF-α and interferon-γ (IFN-γ) may represent potent inducers of lymphocyte migration in the skin.[28] The local release of TNF-α may be of great importance in initiating and maintaining the inflammatory response in the skin. In addition, an increased production of TNF-α by keratinocytes after an initial stimulation leads to an immobilization of Langerhans' cells.[29] Therefore, a substantial number of antigen-presenting cells will remain in the skin where they can interact with specific infiltrated T lymphocytes.[30] The precise role of IL-1α and TNF-α in the outcome of the immune status of hosts infested with ticks is not known. However, it has been shown that adult *Dermacentor andersoni* salivary gland extract partially inhibits *in vitro* cytokine (IFN-γ, IL-1, TNF-α) production by mononuclear cells.[31] Due to positive staining of TNF-α and IL-1α on some mononuclear cells at the tick feeding site of non-resistant hosts, the question arises as to whether TNF-α and/or IL-1α may be detrimental to BALB/c mice in mounting an efficient resistance to tick feeding.

According to our results, it is tempting to predict a local antigen presentation to $CD4^+$ T cells, which becomes activated and may release cytokines such as IFN-γ. This event may explain the cutaneous delayed-type hypersensitivity (DTH) observed in re-infested BALB/c mice,[5] because $CD4^+$ T cells are believed to be effector cells which mediate the DTH response.[32–34] In addition, *in vivo* ICAM-1 induction accompanies T-cell-mediated hypersensitivity reactions in human skin.[35] Importantly, this DTH response is not sufficient to protect the mouse against re-infestation.

In conclusion, this study shows a selective infiltration of T cells in the skin of mice infested with nymphs of *I. ricinus* ticks, with local production of TNF-α and, to a lesser extent, IL-1α by

mononuclear cells in non-resistant BALB/c mice. This immunological response to tick feeding is also accompanied with an increase of cells expressing Ia antigens and ICAM-1, which may partially mediate the extravasation of inflammatory cells described in the skin of infested BALB/c mice.[5]

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PUBLICATION III

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IFN-γ, IL-2, and IL-4 mRNA Expression in the Skin and Draining Lymph Nodes of BALB/c Mice Repeatedly Infested with Nymphal *Ixodes ricinus* Ticks

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The skin and draining lymph nodes of BALB/c mice were examined for IFN-γ, IL-2, and IL-4 mRNA expression by *in situ* hybridization in three successive infestations with nymphal *Ixodes ricinus* ticks. IFN-γ and IL-2 mRNA positive cells were readily detected in lymph node sections during primary antigenic stimulation (72 hr post-tick attachment), whereas hybridization with IL-4 probe yielded no or only a faint positive signal. No changes in the cytokine pattern were observed in lymph node sections from reinfested mice, with IL-4 mRNA always being expressed to a lesser extent than IFN-γ and IL-2 mRNA. Seventy-two hours post-tick attachment in primary infestation, some infiltrating cells in the skin were positive for IFN-γ and IL-4 mRNA, but not for IL-2 mRNA. In skin sections of reinfested mice, mRNA coding for IFN-γ, IL-2, and IL-4 were detected in infiltrating cells. Cells positive for IL-4 mRNA were lower in number than those positive for IFN-γ and IL-2 mRNA. A significant decrease in the number of IL-4 mRNA positive cells in the tertiary infestation was noted. All together, these results indicate that *I. ricinus* nymphal ticks antigens are able to elicit expression of IFN-γ, IL-2 mRNA and to a lesser extent IL-4 mRNA in both skin and draining lymph nodes. In addition, repeated infestations with ticks led to strong expression of IFN-γ and IL-2 mRNAs in the skin that may be correlated with previous observations showing the occurrence of cutaneous delayed-type hypersensitivity in tick-infested mice. Notably, the cytokine pattern observed in the skin and draining lymph nodes is not associated with a protective immune response in mice against *I. ricinus* nymphal ticks infestations.

INTRODUCTION

The immunological basis of the acquisition of resistance to tick feeding has been partially established. Specific anti-tick antibodies (IgG) have been demonstrated playing a role in tick-resistance in guinea pigs and rabbits (1–3). The cellular immunity also plays a key role in the acquisition of resistance in some laboratory animals. Thus, a cutaneous basophil hypersensitivity occurred in resistant guinea pigs infested with *Dermacentor andersoni* larvae (4). Treatment of rabbits with cyclosporin A during infestations with *Ixodes ricinus* ticks attenuated the effects of the resistance of the hosts against the ectoparasites (5). Moreover, such treatment blocked the immediate cutaneous (type I) reaction in infested rabbits and led to a decreased delayed (type IV)

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hypersensitivity to tick antigen (6). On the other hand, the alternative complement pathway was also shown to be involved in the acquisition of resistance to ticks (7).

The study of the local inflammatory response related to the immune status of the murine hosts to tick feeding yielded several reports. Thus, it has been suggested that mast cells and eosinophils might participate in the acquisition of resistance to *D. variabilis* larvae in BALB/c mice (8). Furthermore, genetically mast cell-deficient *W/W*^{v} mice were not able to mount a protective immune response when infested with larval *Haemaphysalis longicornis* ticks (9). However, mast cell-deficient *W/W*^{v} mice acquired resistance after repeated infestations with *D. variabilis* larvae (10), and basophils were suggested to be involved in the acquisition of resistance (11). Our previous studies showed that BALB/c mice infested with nymphal *I. ricinus* ticks failed to acquire resistance despite the marked cellular infiltrate in the skin, including neutrophils, eosinophils, and basophils (12). A higher number of degranulated mast cells was observed in reinfestations and the skin of infested mice became sensitized to tick antigens (immediate and delayed type). Therefore, the immunological mechanisms underlaying the immune response to ticks in murine hosts need to be investigated more deeply.

In the mouse, different patterns of cytokines have been shown to lead to different functional properties resulting in the control or the promotion of some diseases (13, 14). T helper lymphocytes in mice have been divided into two subsets according to the profile of cytokines they produced. The TH1 subset produces IFN-γ and IL-2 and is involved in cell-mediated immune responses (delayed-type hypersensitivity (DTH) and macrophages activation). The TH2 subset produces IL-4, IL-5, and IL-10 and assists in antibody production for humoral immunity. Most of the studies regarding the cytokine profile were done using experimental systems with intracellular microorganisms or parasitic helminth models.

In the present paper, *in vivo* study was undertaken to look for IFN-γ, IL-2, and IL-4 mRNAs using *in situ* hybridization in skin and draining lymph node sections of BALB/c mice undergoing three repeated infestations with nymphal *I. ricinus* ticks.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Iffa-Credo (Arbresle, France). They were between 8 to 12 weeks old at the time of infestations.

Ticks

I. ricinus nymphal ticks were reared in our laboratory as previously described (15).

Infestations

Fifteen *I. ricinus* nymphs per mouse were placed within a plastic capsule glued to the skin as previously described (12). Three successive infestations were done and no skin site was infested more than once. The duration of each infestation was between 4 and 7 days. Mice were given 7 days free of ticks between successive infestations.

Tissue Preparation

Skin biopsies, including attached ticks, were taken 72 hr post-tick attachment. Axillary lymph nodes draining tick attachment sites were carefully removed. Skin biopsies and lymph nodes were embedded in Optimal Cutting Temperature compound (Bayer-Pharma, Zürich, Switzerland), then frozen in isopentane chilled in liquid nitrogen and stored at −70°C.

Probes

All probes used were synthetic oligonucleotide probes, labeled at the 5′ end with digoxigenin. The cDNA for murine IL-2 and IL-4 (30 bases each) were complementary to murine mRNAs cytokines and were purchased from British Biotechnology Products (Oxon, UK). The IFN-γ probe was complementary to bases 505 to 534 of mouse IFN-γ (16) and was obtained from Microsynth (Windish, Switzerland).

In Situ Hybridization

Cryostat sections (5-μm thick) were placed on poly-L-lysine-coated slides (17) and allowed to air dry for 1 hr. The ultrapure water used for cleaning slides and for preparation of hybridization solutions was treated with 0.1% diethylpyrocarbonate (Sigma) and allowed to stand at room temperature overnight before autoclaving.

Skin and lymph node sections were fixed in 4% paraformaldehyde for 20 min at 4°C. After several washes in 0.1 *M* phosphate buffer saline, pH 7.2, free amino groups were acetylated by treatment with 0.25% acetic anhydride in 0.1 *M* triethanolamine, pH 8. Sections were prehybridized for 1 hr at 37°C in hybridization buffer consisting of 2× SSC (1× SSC = 150 m*M* NaCl, 15 m*M* trisodium citrate, pH 7), 30% of deionized formamide (Gibco BRL, Basel, Switzerland), 1× Denhardt's solution, 125 μg/ml sheared denaturated salmon sperm DNA (Gibco BRL). Slides were then drained and sections covered with 50 μl of hybridization buffer containing DNA probe at a concentration of 0.5 mg/ml. Sections were covered with siliconized coverslips, sealed with rubber cement, and hybridized overnight at 37°C.

Posthybridization washing was performed in decreasing concentration of SSC (4×, 2×, and 0.2×) with 30% formamide at 37°C. After 15 min washing at room temperature in Tris-buffered saline (TBS) (0.05 *M* Tris–HCl, 0.15 *M* NaCl, 2 m*M* $MgCl_2$, 0.1% bovine serum albumin), pH 7.6, containing 0.1% Triton X-100, the sections were incubated with sheep anti-digoxigenin–alkaline phosphatase conjugated antibodies (British Biotechnology Products, Oxon, U.K.) diluted 1/600 in TBS. Alkaline phosphatase activity was demonstrated using β-chloroindolyl-phosphate-nitroblue tetrazolium (British Biotechnology Products) as substrates for 3–6 hr. Slides were then counterstained with Mayer's haematoxylin solution (Fluka, Buchs, Switzerland).

Sections incubated with Rnase A (Boehringer-Mannheim, Rotkreuz, Switzerland) (0.1 mg/ml in 2× SSC, 10 m*M* $MgCl_2$) for 1 hr at 37°C and the application of the hybridization solution, without probe, served as negative control.

Microscopic Evaluation

Two sections per mouse for a total of three mice per infestation were viewed with an Olympus Vanox-S microscope. A total area of 1.24 mm^2 per skin cryostat section was examined.

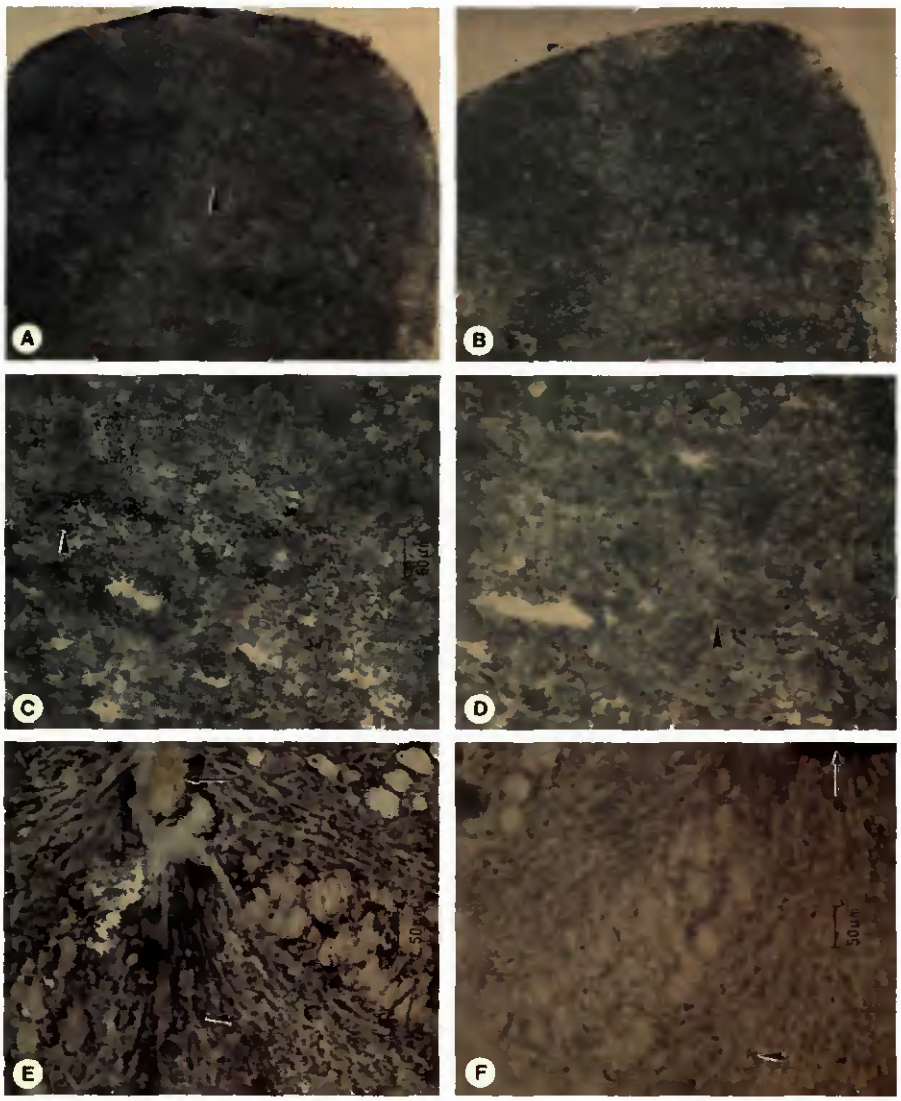

FIG. 1. *In situ* hybridization of mice draining lymph nodes (A–D) and skin sections (E, F) in primary (A, B) and tertiary infestation (C–F), 72 hr post-tick attachment. IFN-γ (A, C) and IL-4 mRNAs (B, D) positive lymphocytes (arrowhead). Positive signal for IFN-γ (E) and IL-4 mRNAs (F) in infiltrating cells (arrowheads) in the dermis near the tick's rostrum (arrow).

RESULTS

Primary Infestation

In draining lymph nodes, IFN-γ and IL-2 mRNAs were detected mainly in the paracortex area (Fig. 1A). No signal (Fig. 1B) or a weak positive one confined in

some lymphocytes in the paracortex was found for IL-4 mRNA in lymph nodes sections.

In the skin, a positive signal for IFN-γ mRNA was found in some infiltrating mononuclear cells in the dermis, primarily near the tick rostrum. No signal was obtained for IL-2 mRNA, but a few cells were positive for IL-4 mRNA (not shown).

Secondary and Tertiary Infestations

There was an obvious increase in the size of lymph nodes of mice draining the tick attachment sites. Lymph nodes sections revealed an extension of the paracortical area. IFN-γ and IL-2 mRNA were readily detected in lymph node sections of reinfested mice. Numerous positive lymphocytes for IFN-γ and IL-2 mRNAs were generally located in vessels packed with lymphocytes in the paracortical area. A faint positive signal for IL-4 mRNA was detected in lymph node sections, contrasting with the strong positive signal obtained for IFN-γ and IL-2 mRNAs (compare Figs. 1C with 1D). In addition, cells positive for IL-4 mRNA remained at low abundance and were again found mainly in the paracortical area.

Overall results of *in situ* hybridization in skin cryostat sections are summarized in Table 1. Hybridization with IFN-γ probe yielded an intense positive signal in the skin of reinfested mice (Fig. 1E). Infiltrating mononuclear cells positive for IFN-γ mRNA increased in number compared to the primary infestation. Numerous cells were also positive for IL-2 mRNA, and they were generally located near the tick rostrum. The positive signal pattern obtained for IFN-γ and IL-2 mRNAs remained the same in skin sections from the secondary and tertiary infestations. Cells positive for IL-4 mRNA also increased in number in the secondary infestation compared to the primary infestation. However, a lower number of cells appeared to be positive for IL-4 mRNA in the tertiary infestation compared to the secondary infestation.

DISCUSSION

Previous studies in guinea pigs infested with *D. andersoni* larval ticks have revealed that tick antigen injected into the skin was trapped by Langerhans cells and presented

TABLE 1

Cytokines mRNA Positive Cells in Skin Biopsies from BALB/c Mice Infested with *Ixodes ricinus* Ticks

	mRNA positive cells		
Infestations	IFN-γ	IL-2	IL-4
1	±	—	±
2	+++	++	++
3	+++	++	+

Note. −, negative; ±, <5% mRNA positive cells; +, 5–20% mRNA positive cells; ++, 20–50% mRNA positive cells; +++, >50% mRNA positive cells. The cellular infiltrate in skin section (1.24 mm^2) 72 hr post-tick attachment consisted approximately of 60 cells in the primary infestation and 230 cells in reinfestations (12). The relative percentage of positive cells for each probe in skin biopsy was considered in comparison with the total cellular infiltrate.

to T lymphocytes in draining lymph nodes (18, 19). The immune response to *I. ricinus* nymphal ticks observed in BALB/c mice lymph node sections revealed a preferential expression of IFN-γ and IL-2 mRNA during primary antigenic stimulation (72 hr post-tick attachment). IL-4 mRNA was not detected in lymph nodes sections by Day 3 after tick attachment in primary infestation. However, it cannot be excluded that IL-4 mRNA can be detected in lymph nodes at a later stage in the primary infestation. IL-4 mRNA was more readily detected in lymph node sections of restimulated mice. These results pointed out a sequential cytokine mRNA production in tick-infested mice. IL-4 mRNA was shown to be produced *in vivo* after primary picryl-chloride-sensitization, and elevated IL-2 mRNA preceded IL-4 mRNA (20). The fact that IL-2 mRNA producing cells were detected before IL-4 mRNA may result in the requirement of IL-2 for IL-4 production (21, 22). Evidence has been reported that IL-2 plays a major role in the differentiation of resting T cells into cells that are capable of secreting IL-4 when they are restimulated (21). Also, IL-4-secreting cells in keyhole limpet hemocyanin primed lymph nodes cells were detected in higher proportions after one cycle of *in vitro* antigen restimulation (21). Our results support the view that cells responsible for IL-4 production may require an additional priming step with antigen before starting production of IL-4 mRNA (21). The expression of IL-4 mRNA remained low in secondary and tertiary infestations. This might result from the activity of IFN-γ because it was shown to downregulate the TH2 cell growth (23). Both IL-4 and IFN-γ mRNAs were weakly detected in the skin of mice 72 hr in the primary infestation. The possibility that other cell types apart from T cells could produce IL-4 is not excluded. Mast cells and basophils, already described in the skin of tick-infested BALB/c mice (12), were detected as sources of IL-4 (24, 25). Changes in the number of cells positive for IL-4 mRNA were noted in the skin of reinfested mice. IL-4 mRNA positive cells were detected in low numbers in the skin of mice undergoing two manifestations with ticks, and an obvious decrease in number occurred in the tertiary infestation. One possible explanation is that repeated exposures to ticks led progressively cells to produce IFN-γ and IL-2 mRNA in the skin of mice. Host immune response could also be modulated via pharmacological substances such as E serie prostaglandins (PGE2) present in the saliva of several tick species (26, 27). Such tick's modulation may allow the detection of IL-4 mRNA as much as the host is submitted to a limited cycle of infestation because PGE2 was demonstrated to tip the balance in favor of a TH2-type response (28). This event could be beneficial to ticks, because IL-4 was demonstrated to have an important anti-inflammatory role (29, 30). Our previous studies have demonstrated that BALB/c mice repeatedly infested with nymphal *I. ricinus* ticks failed to acquire resistance (12). The skin of reinfested mice showed a prominent infiltration of neutrophils, eosinophils, and basophils that peaked 72 hr post-tick attachment. Furthermore, the skin of infested mice became sensitized against tick antigens (immediate and delayed type). IFN-γ and IL-2 mRNA were both readily detected in the skin and lymph nodes sections of reinfested mice. This observation is consistent with the cutaneous DTH reaction previously described (12). DTH was shown to be induced by cells of the TH1 subset and IFN-γ can mediate lymphocyte infiltration in the sites of cutaneous DTH in rats (31, 32). Moreover, DTH induced in mice by injections of TH1 clones were partially inhibited by anti-IFN-γ antibodies (33). We previously noted an increase in number of $CD4^+$ and $CD8^+$ T cells in the skin of reinfested mice (Mbow *et al.*, submitted for publication), and the detection of

IL-2 mRNA suggests that local proliferation of T cells in the skin might have occurred in reinfested mice following local tick antigen presentation to T cells. An increase in proliferation of T cells in the skin was observed upon systemic rIL-2 administration in the mouse model (34). In our model, there is no production of anti-tick antibodies (unpublished observations). The occurrence of DTH reaction excluded specific antibodies production (35). The production of specific anti-tick antibodies in murine model may be of central importance in allowing the hosts to mount a protective immune response. Specific anti-tick antibodies may interfere with tick feeding leading to a smaller bloodmeal taken by the ectoparasites.

In conclusion, this study shows a differential cytokine mRNA production in the skin and draining lymph nodes of BALB/c mice repeatedly infested with nymphal *I. ricinus* ticks. A large number of cells positive for IFN-γ and IL-2 mRNA characterized the local immune response after two infestations, whereas draining lymph nodes of reinfested mice do not show any changes in the cytokine mRNA patterns, with IFN-γ and IL-2 mRNA being expressed in much more lymphocytes than IL-4. It must be noted that this cytokine pattern does not confer a protective immune response in BALB/c mice against *I. ricinus* nymphal ticks infestations.

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RESULTATS NON PUBLIES

Ability of IL-4-deficient mice (IL-4$^{-/-}$) to acquire transient resistance against nymphal *Ixodes ricinus* ticks.
(Unpublished results)

SUMMARY

The acquisition of resistance against *I. ricinus* nymphal ticks was studied in IL-4 gene targeted mice and in their IL-4 sufficient counterparts by evaluating the mean weights of engorged nymphs and the duration of bloodmeal in the 3 successive infestations. The mean weights and the duration of bloodmeal of nymphs engorged on control mice do not vary in three successive infestations, pointing out the inability of these mice to acquire resistance. Nymphs engorged on IL-4$^{-/-}$ mice showed a significant decrease in their mean weigths only in the secondary infestation. In the tertiary infestation, values of the mean weights reached the levels of that obtained in the primary infestation. The histological examination of the tick fixation sites was done in the tertiary infestation, 3 days post tick attachment. A marked infiltration of eosinophils was noted in the skin, whereas neutrophils and a few eosinophils were the main cellular types infiltrating the skin of IL-4$^{-/-}$ mice. Our results showed a transient resistance acquired by IL-4$^{-/-}$ mice, that became abolished up to two infestations with this ectoparasite.

INTRODUCTION

The acquisition of resistance of murine hosts against immature stages of ticks has been mainly correlated with the nature of the cellular infiltrate in the skin. Thus, mast cells, eosinophils and basophils were considered as the most important cellular types that play a pivotal role in the elaboration of the protective immune response in the murine hosts to ticks' feeding (Den Hollander and Allen, 1985a, 1985b; Matsuda et al., 1985; Steeves and Allen, 1990). However, it has been demonstrated that BALB/c mice failed to acquire resistance against nymphal *I. ricinus* ticks despite the marked infiltration of eosinophils and basophils in the skin (Mbow et al., 1994).

Cytokines represent a group of key molecules that regulate the process of the immune response to parasitic infection (Sher and Coffman, 1992). In the mouse, the outcome of the immune status to these infections depends on the profile of the cytokines produced by T lymphocytes. Thus, CD4^{+} T cells were divided into 2 subsets (TH1 and TH2) according to the cytokines produced (Mosmann et al., 1986). TH1 subset produces IFN-γ and IL-2, whereas TH2 subset produces IL-4, IL-5 and IL-10. Trials to investigate the mediation of cytokines in the protective immune response to ticks yielded very few reports. Treatment of rabbits with IL-2 was shown to enhance the expression of resistance against *I. ricinus* adults ticks

(Schorderet and Brossard, 1994). Indirect evidence of the involvement of cytokines in the immunity to ticks was also provided since IgE antibodies were shown to be necessary to the acquisition of resistance against larval *Haemaphysalis longicornis* tick in mice (Matsuda et al., 1990). IL-4 was shown to have diverse effects on T and B cells, notably in promoting the production of IgE antibodies and in directing the development of a TH2 response *in vitro* (Snapper and Paul, 1987; Swain, 1993). The study of the local immune response of BALB/c mice to nymphal *I. ricinus* ticks by *in situ* hybridization showed a predominance of cells expressing IFN-γ, IL-2 mRNA in the tertiary infestation (Mbow et al., in press). In this study, attempts were made to analyse the acquisition of resistance in mice genetically deficient in IL-4 to probe the importance of this cytokine in the host immunity to *I. ricinus* ticks.

MATERIALS AND METHODS

Ticks

I. ricinus ticks were reared in our laboratory as previously described (Graf, 1978).

Mice

C57BL/6 IL-4-deficient mice (IL-4$^{-/-}$) (Kopf et al., 1993) and the IL-4-wild type were kindly provided by Dr. G. Le Gros (Ciba-Geigy, Basel, Switzerland). The mice were between 10 to 12 weeks old at the time of infestations.

Assessment of acquired resistance to ticks

Mice were infested successively 3 times with *I.ricinus* nymphal ticks as previously described (Mbow et al., 1994). Briefly, 15 ticks per mouse were placed in a plastic capsule glued onto the skin. Mice were kept seven days free of ticks between successive infestations. Engorged ticks were allowed to moult in order to separate male and female populations as previously described (Graf, 1978). The following parameters were used to assess the acquisition of resistance: the duration of bloodmeal and the weights of engorged ticks.

Histology

Skin biopsies of infested mice were taken 72 hr post-tick attachment in the tertiary infestation from control (n=3) and IL-4$^{-/-}$ mice (n=3), and processed for histological examination as previously described (Mbow et al., 1994). Sections were stained with Giemsa solution (Merck, Switzerland) and examined under an Olympus Vanox-S microscope.

Statistical analysis

A Mann Whitney *U* test was used to compare the data obtained for the assessment of the acquired resistance to ticks.

RESULTS

The weights and durations of the bloodmeal do not differ during the 3 infestations in control mice (Table 1). Histological examination of the tick fixation sites in tertiary infestation revealed a marked infiltration of eosinophils in the dermis, many of them being degranulated. A few mast cells were also detected, and neutrophils, monocytes and lymphocytes remained in low abundance (not shown).

In IL-4$^{-/-}$ mice, it was noted a decreased in weights of engorged male and female nymphs in the secondary infestation compared to the primary infestation ($P< 0.05$; $P< 0.03$) (Table 2). In tertiary infestation, the weights of engorged ticks increased compared to the previous one. No changes in the duration of the bloodmeal were observed in the 3 infestations. The histological examination of the skin in the tertiary infestation showed an eosinophilic infiltration, but in a lesser extent than in control mice. On the contrary, mast cells, neutrophils, monocytes and lymphocytes markedly infiltrated the skin compared to control mice.

Table 1. Development of nymphal *Ixodes ricinus* ticks for 3 infestations on 4 control mice (IL-$4^{+}/^{+}$).

INFESTATIONS	Duration of bloodmeal (Days)		Weight of engorged tick (mg)	
	Male	Female	Male	Female
1	4.40 ± 0.73	4.60 ± 0.50	2.85 ± 0.25	4.46 ± 0.81
2	4.53 ± 1.48	4.76 ± 1.08	2.58 ± 0.30	4.27 ± 0.50
3	5.33 ± 1.52	4.50 ± 0.70	2.70 ± 0.26	4.70 ± 0.50

Table 2. Development of nymphal *Ixodes ricinus* ticks for 3 infestations on 4 IL$4^{-}/^{-}$ mice.

INFESTATIONS	Duration of bloodmeal (Days)		Weight of engorged tick (mg)	
	Male	Female	Male	Female
1	4.31 ± 0.40	4.59 ± 0.43	2.97 ± 0.19	4.60 ± 0.60
2	3.75 ± 0.58	4.00 ± 0.73	2.59 ± 0.33*	4.19 ± 0.60†
3	4.00 ± 0.5	4.31 ± 0.60	2.70 ± 0.26	4.70 ± 0.44

* $P < 0.05$

† $P < 0.03$

DISCUSSION

Tick-resistant BALB/c mice showed significant reductions in percentages of engorged larvae and in mean weights of fed *Dermacentor variabilis* ticks (Den Hollander and Allen, 1985). Due to the marked infiltration of mast cells and eosinophils in the skin of reinfested mice, several authors suggested that these cell types may be important for the expression of resistance to ticks (Den Hollander and Allen, 1985; Hushio et al., 1993). On the other hands, it was demonstrated that specific IgE anti-tick antibodies were necessary in the acquisition of resistance against *H. longicornis* ticks (Matsuda et al., 1990). In our study, IL-4-deficient mice were able to acquire a transient resistance against nymphal *I. ricinus* ticks, as shown by the significant decrease in mean weights of engorged ticks. Ticks allowed to feed repeatedly on their IL-4 sufficient counterparts do not show any changes in their mean weights. The histological examination of the tick-attachment sites in control mice revealed a marked infiltration of eosinophils, many of them being degranulated. These results provided further evidence that the phenomenon of the acquisition of resistance against ticks in mice may not be only linked to the nature of the cellular infiltrate. In addition, this marked infiltration of eosinophils could be a result of a local TH2 response beneficial to ticks in C57BL/6 mice. It has been shown that subcutaneous injection of IL-4 recombinant protein in mice resulted in the accumulation of eosinophils (Tepper et al., 1992). The mechanism of this eosinophilic infiltration was IL-5-independant because mice pretreated with anti-IL-5 antibodies, at doses previously shown to inhibit eosinophilia in helminth-infected mice, also showed a marked infiltration of eosinophils in IL-4 treated mice. The disruption of the IL-4 gene in mice blocked the TH2 response in *Nippostrongylus brasiliensis*-infected mice (Kopf et al., 1993). We showed that IL-4-deficient mice developped a resistance against ticks only in the secondary infestation, the mean weights of fed ticks in the tertiary infestation do not significantly differ to those in the primary infestation. Therefore, IL-4 is not indispensable in the acquisition of resistance against ticks, at least when the host are subjected to a limited cycle of infestations. The exact mechanisms that mediate the decreased bloodmeal uptake by ticks are not known. However, one can predict that, in the secondary infestation, tick stimulation may allow cells to produce IFN-γ due to the lack of the antagonist effect mediated by IL-4, which is known to inhibit the production of IFN-γ (Sher and Coffman, 1992). IFN-γ was shown to be involved in cell-mediated immunity and to be a proinflammatory cytokine (Halloran, 1993). On the other hand, the possibility that a progressive replacement of IL-4 by another cytokine having similar properties *in vivo* upon chronic tick antigenic stimulation is not excluded. It has been shown, for example, that IL-13 and IL-4 shared several functional properties

(Zurawski and de Vries, 1994). Moreover, it was observed that IL-4 deficient mice were able to generate a TH2 response after 2 infestations with *N. brasiliensis* (Müller et al., 1993).

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5. DISCUSSION GENERALE ET CONCLUSIONS

Les infestations répétées des souris BALB/c avec des nymphes d'*I. ricinus* n'affectent pas le développement des tiques. En effet, le poids moyen des ectoparasites engorgés, le pourcentage de fixation et le temps de mue ne varient pas pendant 3 infestations successives (Mbow et al., 1994a).

En primoinfestation, le derme est faiblement infiltré de neutrophiles et de cellules mononucléées, avec des lymphocytes T $CD4^+$ et $CD8^+$. Quelques mastocytes sont en voie de dégranulation par l'action probable d'enzymes salivaires injectées dans la peau (Geczy et al., 1971). La libération de médiateurs tels que l'histamine, l'héparine et la sérotonine par les mastocytes initie sans doute le processus inflammatoire local. La nutrition des tiques serait favorisée par l'action anticoagulante et par l'augmentation de la perméabilité vasculaire dues à ces substances (Weitzman et al., 1985; Scully et al., 1986). Des cytokines peuvent être libérées par les mastocytes, le TNF-α en est un exemple (Gordon et Galli, 1990). Ceci constitue un évènement important dans la réaction inflammatoire locale, car cette cytokine assure en partie le recrutement des neutrophiles, monocytes et lymphocytes (Zhang et al., 1992; Larsen et al., 1990). Nous avons détecté du TNF-α et de l'IL-1α au niveau des kératinocytes de l'épiderme et des CDD chez les souris infestées par les tiques, suggérant un rôle important de ces cytokines dans la mise en place de la réponse inflammatoire locale. Leur présence dans l'épiderme résulterait du dommage mécanique causé par le rostre des tiques. En effet, les kératinocytes réagissent à la moindre perturbation de la barrière cutanée en produisant de l'IL-1α et du TNF-α; par effet autocrine, celles-ci amplifient leur sécrétion (Luger et Schwartz, 1990).

Dans la peau, l'expression des ICAM-1 est limitée aux kératinocytes et aux CDD. Les Ia sont exprimés sur les CL, sur les CDD et sur d'autres cellules mononucléées ayant l'apparence de macrophages. L'intensité de l'expression des ICAM-1 et des Ia dans l'épiderme des souris en primo-infestation est similaire à celle observée dans la peau normale. Il est connu que ces molécules de surface sont exprimées constitutivement sur certaines cellules de la peau (CL, CDD, macrophages, cellules endothéliales des vaisseaux sanguins); la régulation de leur expression est contrôlée par des cytokines telles que le TNF-α et l'IFN-γ (Glimcher et Kara, 1992; Bevilacqua, 1993). Nous avons localisé les ARNm de l'IFN-γ et de l'IL-4 dans quelques cellules du derme; les ARNm de l'IL-2 ne sont pas détectés. Cette observation suggère que les lymphocytes T du site de fixation des tiques chez les souris en primoinfestation ne sont pas activés (Mbow et al., 1994c). D'autres

types cellulaires que des lymphocytes T produiraient de l'IL-4, les mastocytes et les basophiles par exemple (Galli et al., 1991).

Les tiques injectent des antigènes salivaires pendant leur repas sanguin (Kaufman, 1989). Chez les cobayes infestés par des larves de *D. andersoni*, les CL digèrent et présentent les antigènes aux lymphocytes T dans les ganglions de drainage (Nithuithai et Allen, 1985). Dans ce système, l'épiderme est complètement altéré, avec une formation de cavités remplies de cellules inflammatoires (Allen, 1973). Chez les BALB/c infestées par des nymphes d'*I. ricinus*, l'épiderme n'est pas modifié, le rostre de la tique pénètre profondément dans le derme (Mbow et al., 1994a). Les CDD pourraient induire la réponse immune primaire dans notre système. En effet, il a été montré récemment, chez l'homme et la souris, que le derme contenait des sous-populations de cellules dendritiques ayant la capacité d'induire une prolifération de lymphocytes T *in vitro* et *in vivo* de manière tout aussi efficace que les CL (Nestle et al., 1993; Lenz et al, 1993; Levin et al., 1993). L'étude des ARNm des cytokines dans les ganglions des souris BALB/c, 3 jours après la fixation des tiques, révèle une production préférentielle de cytokines pendant la réponse immune primaire. Les messagers de l'IFN-γ et de l'IL-2 sont facilement localisés dans les lymphocytes, contrairement à ceux de l'IL-4, absents à ce stade de l'infestation (Mbow et al., 1994c). L'apparition différée de l'IL-4 en deuxième infestation par rapport à l'IL-2 reflète sans doute la nécessité d'une intervention préalable de l'IL-2 pour la production d'IL-4 lors d'une stimulation antigènique primaire (Ben-Sasson et al., 1990; Mohler et Butler, 1990).

Chez les souris réinfestées par les tiques, la réponse inflammatoire est considérablement amplifiée (Den Hollander et Allen, 1985a; Steeves et Allen, 1991). Chez les souris BALB/c infestées par des nymphes d'*I. ricinus*, nous notons une importante infiltration de neutrophiles, d'éosinophiles et de basophiles (Mbow et al., 1994a). Les mastocytes en voie de dégranulation augmentent en nombre, conséquence probable d'une sensibilisation progressive de la peau par les antigènes des tiques. En effet, des réactions cutanées d'hypersensibilité immédiate ont été mesurées après injection d'un extrait antigènique de tiques (Mbow et al., 1994a). L'implication des anticorps IgE dans ces réactions reste à déterminer. Par ELISA et Western blot, nous n'avons pas détecté d'anticorps anti-tiques de classe IgG1 chez des souris réinfestées (résultats non publiés). L'IFN-γ que nous avons localisé dans la peau et dans les ganglions de drainage, inhibe les réponses IgE et IgG1 (Finkelman et al., 1990). Les anticorps IgE n'interviennent pas toujours dans les réactions d'hypersensibilité immédiate. Chez les souris, les lymphocytes T libèrent des facteurs spécifiques à l'antigène pouvant remplacer les IgE dans l'induction de ces réactions (Ptak et al., 1982; Askenase, 1992).

L'intense réaction inflammatoire locale des souris réinfestées avec *I. ricinus* est accompagnée par une forte expression des molécules ICAM-1 et Ia sur un plus grand nombre de cellules. Les cytokines TNF-α, IL-1α et IFN-γ, produites rapidement et en quantité plus importante chez les souris réinfestées, sont sûrement à l'origine de l'expression augmentée de ces molécules. Elles peuvent occasionner la formation d'un gradient de facteurs chimiotactiques (IL-8 par exemple) attirant les leucocytes vers le site enflammé (Warren 1990). L'extravasation des leucocytes à travers les parois endothéliales est sans doute favorisée par les ICAM-1 (Bevilacqua, 1993; Rothlein et al., 1993)

D'autre part, les lymphocytes T $CD4^+$, qui infiltrent le site de fixation des tiques en grand nombre, pourraient être activés par les antigènes salivaires et produire localement des cytokines. Cette hypothèse est d'autant plus plausible que certains préalables au processus de présentation des antigènes sont remplis localement. Aussi bien les Ia que les ICAM-1, intervenant dans ce processus (Dang et al., 1990; Damle et al., 1992; Takahashi, 1993), sont exprimés par les CDD et d'autres cellules mononucléées. De plus, les souris réinfestées développent une DTH cutanée après injection d'un extrait antigènique de tiques (Mbow et al., 1994a). L'induction d'une DTH chez les souris est contrôlée par la libération de sérotonine, plutôt que par celle de l'histamine (Askenase, 1992). Notons que les mastocytes ne représentent pas la seule source de sérotonine, car les basophiles qui infiltrent aussi le site de fixation des tiques chez les souris réinfestées en produisent aussi (Askenase, 1992). La phase effectrice d'une DTH requiert la participation des T $CD4^+$ (Askenase, 1992), plus précisément la sous-population TH1 chez les souris (Cher et Mosmann, 1987). La prolifération locale des lymphocytes T a été démontrée chez des animaux développant une DTH de type tuberculinique (Fritz et al., 1990). Elle n'est pas exclue chez les souris BALB/c réinfestées, d'autant plus que les ARNm d'IL-2 ont été visualisés localement. Les T $CD8^+$, infiltrant le site de fixation des tiques pourraient aussi être impliqués dans les réactions de DTH. En effet, le profil des cytokines synthétisées par ces lymphocytes activés est similaire à celui des TH1 (Fong et Mosmann, 1990).

Les messagers de l'IFN-γ et de l'IL-2 sont de plus en plus intensément exprimés localement chez des souris BALB/c au fil des infestations par *I. ricinus* (Mbow et al., 1994c). Nous avons remarqué une diminution des cellules positives pour les ARNm de l'IL-4 en troisième infestation. Lors des deux premières infestations, les tiques pourraient moduler la production de cytokines par des substances salivaires comme les prostaglandines E2 (Ribeiro et al., 1992) qui favorisent l'expansion des TH2 (Phipps et al., 1991). Les types de CPAg et la disponibilité de cofacteurs, notamment d'IL-1, peuvent aussi conditionner le profil

des cytokines produites par les sous-populations de T $CD4^+$ (Weaver et al., 1988; Chang et al., 1990; Gajewski et al., 1991; Schmitz et al., 1993). Ces auteurs suggèrent qu'une prolifération optimale des TH2 nécessiterait la présence des lymphocytes B comme CPAg, tandis que l'expansion des clones TH1 impliquerait celle de CDD ou de macrophages. Chez les souris BALB/c réinfestées, les seuls types cellulaires pouvant jouer le rôle de CPAg dans la peau demeurent les cellules dendritiques et les macrophages, aucune infiltration de lymphocytes B CD45R n'étant observée (Mbow et al., 1994b). L'évolution décrite du profil des cytokines dans la peau n'est pas observée dans les ganglions de drainage chez les souris réinfestées. En effet, les lymphocytes positifs pour les ARNm de l'IFN-γ, de l'IL-2 et de l'IL-4 ne varient pas en nombre, montrant qu'une stimulation répétée par les antigènes des tiques provoque plutôt un profil de cytokines mixte. L'intervention des T $CD8^+$ reste à déterminer. La présentation des antigènes par d'autres types de CPAg expliquerait cette situation. Les lymphocytes B ganglionnaires, en plus des cellules dendritiques et des macrophages, pourraient intervenir dans ce processus. Plusieurs observations confortent ces hypothèses. Par exemple, la susceptibilité des souris BALB/c aux infections à *Leishmania major* est associée à une forte production d'IL-4 par les lymphocytes T (Heinzel et al., 1991). Ces souris développent une résistance après déplétion des lymphocytes B *in vivo*, bloquant ainsi probablement l'activation des TH2 (Sacks et al, 1984).

Il a été proposé que des situations de réponse mixte TH1 et TH2 conduiraient à une inhibition de la production d'anticorps par l'IFN-γ et à une absence de DTH due à l'antagonisme de l'IL-4 sur la prolifération des TH1 (Mosmann et Coffman, 1989). Si le premier cas de figure semble se vérifier chez les BALB/c infestées par des tiques, où effectivement la production des anticorps IgG est abolie (observations non publiées), il n'en reste pas moins que les réactions de DTH cutanées ne sont pas totalement inhibées.

En conclusion, l'absence de résistance des souris BALB/c ayant subi des infestations répétées avec des nymphes d'*I. ricinus* est accompagnée d'une production cutanée de TNF-α et d'une forte expression des ARNm pour l'IFN-γ et l'IL-2. Le profil des cytokines des ganglions de drainage est caractérisé par l'expression des ARNm pour l'IFN-γ, l'IL-2 et l'IL-4. L'IFN-γ et le TNF-α représentent des cytokines proinflammatoires importantes (Halloran, 1993; Vassalli, 1992). Les effets induits par le TNF-α sont augmentés par l'IFN-γ (Tsujimoto et al., 1986; Barker et al., 1990). Ces cytokines favoriseraient la nutrition des tiques. Les adultes d'*I. ricinus* se nourrissent mieux sur des lapins traités avec la protéine recombinante TNF-α que sur des animaux contrôles (Schorderet, 1993). S'il est connu que certains composants salivaires de la tique ont un effet antihémostatique

(Ribeiro et al.,1985), il apparait aussi que la nature de la réponse immunitaire locale induite par *I. ricinus* chez les souris BALB/c favoriserait aussi les tiques dans leur développement. L'inhibition de la différenciation des lymphocytes B ganglionnaires en plasmocytes par l'action de l'IFN-γ (Finkelman et al., 1990) favoriserait aussi les ectoparasites. Les souris ne produisent pas d'anticorps IgG anti-tiques (résultats non publiés) contrairement à d'autres systèmes murins où une résistance a été observée (Matsuda et al., 1990). La neutralisation *in vivo* des cytokines IFN-γ ou TNF-α avec des anticorps apporterait une réponse à ces hypothèses.

Les souris C57BL/6, génétiquement déficientes en IL-4 (IL-4$^{-/-}$), acquièrent une résistance transitoire en deuxième infestation (Mbow et al., résultats non publiés). Les souris contrôles (IL-4$^{+/+}$) n'acquièrent pas de résistance contre les nymphes d'*I. ricinus*. L'infiltration intense d'éosinophiles dans la peau suggère une réaction locale faisant intervenir l'IL-4 (Tepper et al., 1992). La déplétion du gène de l'IL-4 bloque la réponse TH2 *in vivo* après des infestations avec des nématodes (Kopf et al., 1993). Néanmoins, de récentes observations ont montré un rétablissement de cette réponse chez les mêmes souris réinfestées avec ces vers (Müller et al., 1993). L'utilisation d'animaux génétiquement déficients afin de comprendre le rôle des cytokines *in vivo* est assez controversée. Les répercussions éventuelles pouvant s'en découler *in vivo* ne sont pas évaluées (Halloran, 1993; Vassalli, 1993). Le fait que des souris IL-4$^{-/-}$ puissent acquérir temporairement une résistance contre les tiques *I. ricinus* mériterait d'être confirmé par une neutralisation *in vivo* de cette cytokine avec des anticorps afin de pouvoir conclure sur son importance dans l'immunité anti-tiques.

Ce travail est une contribution originale à la connaissance des réactions locales induites par les tiques. On peut formuler l'hypothèse que la transmission de microorganismes par ces ectoparasites est influencée par l'intensité et la qualité de cette réponse.

6. RESUME

Les souris BALB/c ne développent pas de résistance contre les nymphes d'*I. ricinus* au cours de trois infestations successives en dépit de l'infiltration importante du derme par des cellules inflammatoires, des neutrophiles, des basophiles et des éosinophiles notamment. De plus, la peau des souris réinfestées est sensibilisée par les antigènes des tiques, ce qui se traduit par le développement de réactions d'hypersensibilité immédiate et retardée dans un test cutané. L'analyse des sous-populations de lymphocytes infiltrant le site de fixation des tiques révèle une absence de lymphocytes B CD45R. Les lymphocytes T $CD4^+$ prédominent sur les $CD8^+$avec un rapport $CD4^+/CD8^+$ qui passe de 2.2 en primoinfestation à 4.7 en troisième infestation.

Par immunohistochimie, nous avons montré au site de fixation des tiques une augmentation de l'expression des ICAM-1 sur les cellules endothéliales des vaisseaux sanguins, les cellules dendritiques et sur quelques cellules mononucléées au cours des infestations. L'expression des Ia sur les cellules infiltrant le derme devient intense chez les souris réinfestées. Les cytokines proinflammatoires IL-1α et TNF-α sont produites localement déjà en primoinfestation dans l'épiderme, et dans le derme par des cellules dendritiques. Le TNF-α est détecté sur des cellules mononucléées infiltrant le derme en réinfestations.

L'analyse du profil des ARNm des cytokines IFN-γ, IL-2 et IL-4 a été réalisée par hybridation *in situ* dans la peau et dans les ganglions axillaires de drainage 3 jours après le début des infestations. En primoinfestation, seuls les ARNm de l'IFN-γ et de l'IL-4 sont détectés dans quelques cellules. Ce profil contraste avec celui observé dans les ganglions de drainage, où seuls les ARNm de l'IFN-γ et de l'IL-2 ont été visualisés.

En deuxième et troisième infestations, l'expression des ARNm des cytokines est dynamique dans les cellules infiltrant la peau. Alors que le pourcentage de cellules positives pour les ARNm de l'IFN-γ et de l'IL-2 reste constant, les cellules positives pour les ARNm de l'IL-4 diminuent. Ainsi, la grande majorité des cellules infiltrant le derme exprime des ARNm pour l'IFN-γ, et l'IL-2.

Au niveau des ganglions de drainage, un profil mixte de cytokines (TH1 et TH2) apparait en reinfestations. Des cellules positives pour les ARNm de l'IFN-γ, de l'IL-2 et de l'IL-4 y sont visualisées. Ce profil de cytokine ne correspond pas à une protection des souris BALB/c contre les tiques.

Les souris C57BL/6, génétiquement déficientes en IL-4 (IL-$4^{-/-}$), acquièrent temporairement une résistance contre les nymphes d'*I. ricinus* en deuxième infestation. Ceci se traduit par une baisse du poids moyen des tiques mâles et femelles nourries sur ces animaux. Au contraire, les souris contrôles (IL-$4^{+/+}$) ne

développent pas de résistance. L'analyse histologique des sites de fixation des tiques sur les souris contrôles, 3 jours après le début de la troisième infestation, révèle une infiltration massive d'éosinophiles, dont certains sont en voie de dégranulation. Cette observation suggère que ces cellules n'interviennent pas dans l'acquisition de la résistance, d'autant plus que la peau des souris IL-4$^{-/-}$ est infiltrée essentiellement par des neutrophiles.

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