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A sequential study of the bovine tuberculin reaction

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SUMMARY

The sequential histopathological and immunocytochemical changes that characterize the tuberculin reaction were studied in 13 cattle experimentally sensitized to *Mycobacterium bovis*, and 14 cattle naturally infected with *M. bovis*. There were two distinct, temporally related patterns of morphological change that were similar for both groups of cattle. The first phase, between 6 hr and 24 hr after the intradermal injection of purified protein derivative (PPD), was characterized by a perivascular aggregation of $WC1^+$ $\gamma\delta$ T cells and neutrophils and the presence of leucocytoclastic vasculitis within the papillary dermis. The second phase of the reaction was characterized by increased numbers of infiltrating $BoCD4^+$ cells, $BoCD8^+$ cells and macrophages, as well as an increase in expression of the interleukin-2 (IL-2) receptor and the ACT2 antigen. Macrophages were the most numerous infiltrating leucocytes between 24 hr and 72 hr after the intradermal injection of PPD. At 72 hr, the reaction was characterized by intense perivascular cuffing with $BoCD4^+$ cells, $BoCD8^+$ cells and macrophages; $\gamma\delta$ T cells and neutrophils were a minor component of the reaction and leucocytoclastic vasculitis was no longer observed. No B cells were detected in the dermis throughout the period of study. The increase in skin thickness was primarily because of inflammatory oedema that was contained within the area by a meshwork of fibrin deposited around the collagen bundles of the reticular dermis.

INTRODUCTION

The tuberculin reaction is considered the prototype T-cell-mediated, delayed-type hypersensitivity (DTH) reaction[1] and the intradermal tuberculin test is the principal method used to diagnose bovine tuberculosis in live cattle. Despite the reliance on this test, there have been no previous immunopathological examinations of the bovine tuberculin reaction. Immunocytochemical examinations of the tuberculin reaction in man revealed that the majority of lymphocytes infiltrating the dermis were $CD4^+$ T cells.[2,3] The purpose of the present investigation was to characterize the sequential histopathological changes occurring at the site of injection of bovine PPD in tuberculin-sensitive cattle and to identify the various leucocyte populations involved in the reaction using immunocytochemical techniques.

MATERIALS AND METHODS

Experimental animals

Group 1. Thirteen cows that were negative to an intradermal tuberculin test were selected from tuberculosis-free herds. They were experimentally sensitized to *Mycobacterium bovis* (*M. bovis*) by a subcutaneous injection of heat-killed *M. bovis* (8–10 mg wet weight, T/88/606, supplied by Dr S. Neill, VRL, Stormont, UK) in Freund's incomplete adjuvant (ICN Biomedical Ltd, High Wycombe, UK) as described previously.[4]

Group 2. Fourteen heifers that were positive to an intradermal tuberculin test were selected from herds with a recent history of infection with *M. bovis*. They were housed in isolation for approximately 6 weeks until the beginning of the study. At the conclusion of the investigation, the lungs and the retropharyngeal, submandibular, mediastinal, mesenteric and hepatic lymph nodes were examined at *post mortem* for lesions of tuberculosis. Suspect lesion material from lymph node and lung was also cultured at 37° for at least 8 weeks on Stonebrinks and Lowenstein–Jensen media to detect the presence of *M. bovis*.

Skin biopsies

Immediately prior to the injection of PPD (0 hr), biopsies of skin in the mid-neck region (control skin) were taken from four cattle in Group 1. A comparative intradermal tuberculin test was performed according to standard methods using avian and bovine tuberculins on the opposite side of the neck. A volume of 0·1 ml of avian PPD (0·5 mg/ml, Central Veterinary Institute, Lelystad, the Netherlands) was injected intradermally in the mid-neck region. A volume of 0·1 ml of bovine PPD (1 mg/ml,

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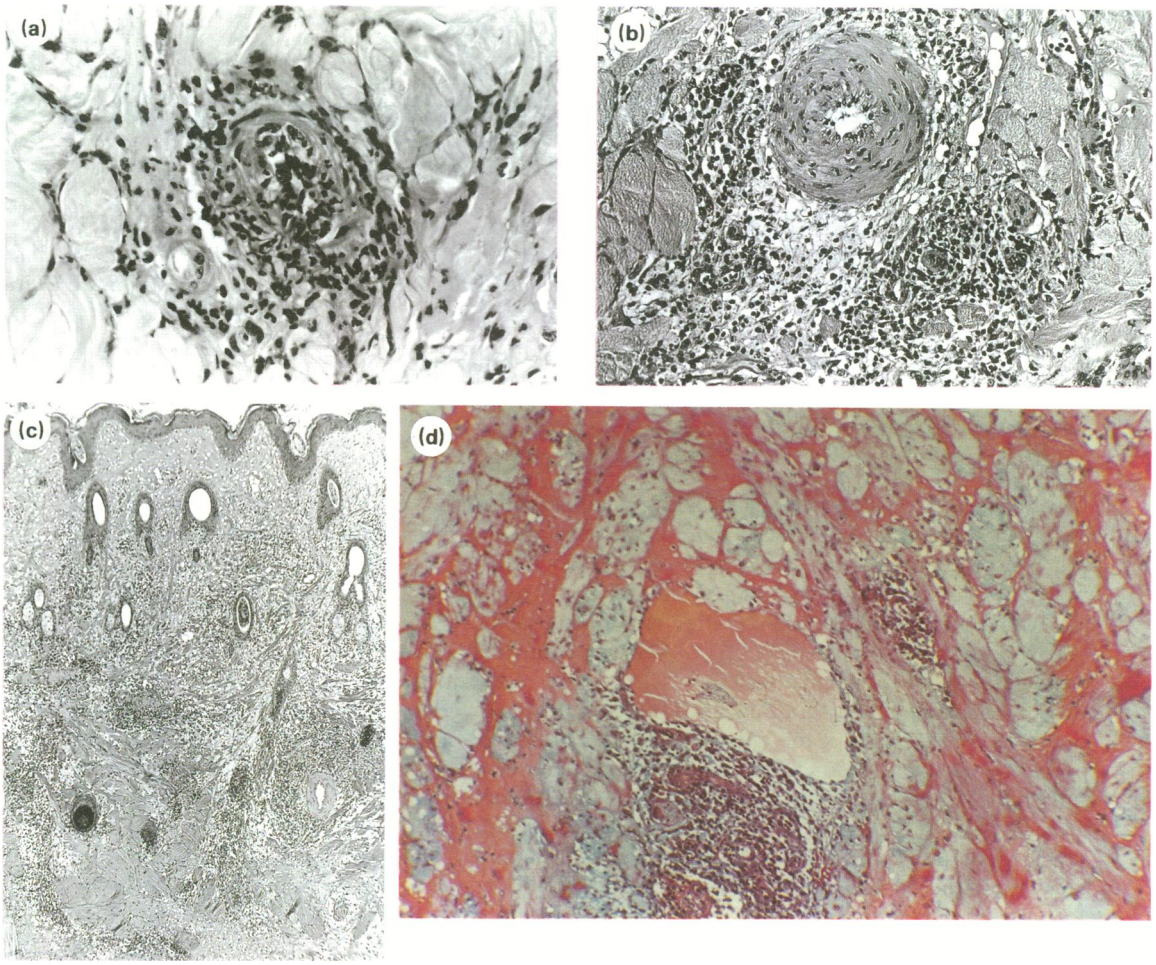

Figure 1. Histopathological examination of the bovine tuberculin reaction. (a) Leucocytoclastic vasculitis affecting an arteriole in the papillary dermis 6 hr after the intradermal injection of bovine PPD (haematoxylin and eosin). (b) Perivascular reaction 72 hr after the intradermal injection of bovine PPD. There is oedema around the arteriole and lymphoid cuffing around adjacent small blood vessels (haematoxylin and eosin). (c) Angiocentric aggregation of lymphocytes and macrophages 72 hr after the intradermal injection of bovine PPD (haematoxylin and eosin). (d) Red-stained intradermal fibrin deposits encircling collagen bundles 72 hr after the intradermal injection of bovine PPD (Martius Scarlet Blue).

Lelystad) was also injected at another site, 12 cm immediately below the avian injection site. Increases in skin thickness were measured immediately prior to biopsy and full-thickness skin biopsies were removed from the centre of the reaction to bovine PPD using an 8-mm biopsy punch after infiltrating the area around the reaction with adrenaline-free lignocaine (Lignavet, C-Vet Ltd., Bury St Edmunds, UK). Each bovine PPD injection site was biopsied on a single occasion only. Biopsies were taken from each of two cattle in Group 1 at 6 hr, 12 hr, 24 hr, 36 hr, 48 hr and 72 hr after the injection of PPD. An individual animal in Group 1 was biopsied at 60 hr. Biopsies were taken from each of two cattle in Group 2 at 6 hr, 12 hr, 24 hr, 36 hr, 48 hr, 60 hr and 72 hr after the injection of PPD.

Each biopsy was sliced in half in a plane perpendicular to its surface. One half was fixed in 10% formol saline, processed through the paraffin series and sections of 5 μm thickness cut from each block were stained with either Harris's haematoxylin and eosin or Martius Scarlet Blue. The other half of each biopsy was embedded in OCT compound, snap-frozen and stored in liquid nitrogen. Frozen sections of 5 μm thickness, cut perpendicular to the surface of the skin were placed onto slides coated with 0·1% poly-L-lysine (BDH Ltd, Poole, UK). The sections were fixed for 10 min in acetone at 4°, protected from desiccation and stored at −70° until required.

The avidin-biotin immunoperoxidase technique

Sections were stained by the avidin-biotin immunoperoxidase technique as described previously.[5] The following monoclonal antibodies were used: anti-BoCD2 (BAQ95A);[6] anti-BoCD4 (CACT138A);[7] anti-BoCD8 (CACT80C);[8] anti-WC1-N1, $\gamma\delta$ T cells (B7A1);[9] anti-IgM, B cells (BAQ44A);[10] anti-GM1, macrophages/neutrophils (DH59B);[11] anti-G1, neutrophils (CH138A);[12] anti-interleukin-2 receptor (IL2-R) (CACT116A)[13] and anti-ACT2 (CACT26A).[14] Endogenous peroxidase was inhibited by flooding the sections with 100 μl of 0·6% solution of hydrogen peroxide in methanol for 5 min. As detection of three of the lymphocyte antigens (BoCD4, WC1 and ACT2) was significantly impaired after methanol-hydrogen peroxide

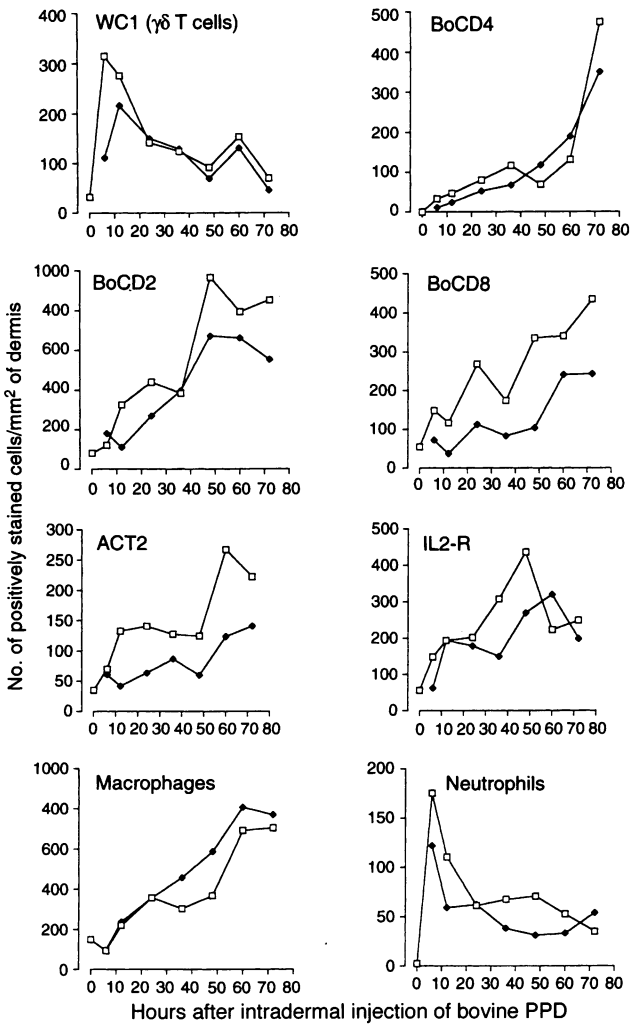

Figure 2. Immunocytochemical examination of the bovine tuberculin reaction. The number of positively stained cells for the WC1 ($\gamma\delta$ T cells), BoCD4, BoCD2, BoCD8, ACT2 and IL2-R antigens and for macrophages and neutrophils, at 0, 6, 12, 24, 36, 48, 60 and 72 hr after the intradermal injection of bovine PPD, in the dermis of animals experimentally sensitized to *M. bovis* (□) and animals naturally-infected with *M. bovis* (◆).

treatment, endogenous peroxidase was inhibited by the application for 15 min of a 1% solution of sodium azide in the sections used for the detection of these antigens.[15] Sections of biopsies from each animal in Groups 1 and 2 taken at 6 and 12 hr after the injection of PPD, were also incubated with 100 μl of biotinylated anti-bovine IgG conjugate (Sigma Chemical Co., St Louis, MO) for 30 min.

Counting of positively stained cells in immunoperoxidase-stained skin sections

The papillary dermis was selected as the counting area as histopathological examination of sections from each biopsy revealed that the cellular infiltration was concentrated mainly within that area. All positively stained cells within the papillary dermis were counted within five microscope fields using the X25 objective. In the case of each section, this approximately constituted the total papillary dermal area. Positively stained cells were counted using a high-resolution, semi-automatic, computer supported image analyser system (VIDS IV, Analytical Measuring Systems, Saffron Walden, UK). The final count at each biopsy time for each section was calculated as the mean of the five fields counted and expressed as the number of cells/mm^2. The number of macrophages present was determined by subtracting the value obtained for the number of neutrophils identified by monoclonal antibody CH138A, from the number of both macrophages and neutrophils identified by monoclonal antibody DH59B.

RESULTS

Increases in skin thickness

The mean increases in skin thickness recorded for the cattle in Group 1 at the time of each biopsy were 3 mm (6 hr), 8 mm (12 hr), 14 mm (24 hr), 5 mm (36 hr), 24 mm (48 hr), 14 mm (60 hr), and 17 mm (72 hr). The mean increases in skin thickness recorded for the cattle in Group 2 at the time of each biopsy were 1 mm (6 hr), 4 mm (12 hr), 6 mm (24 hr), 8 mm (36 hr), 7 mm (48 hr), 9 mm (60 hr) and 13 mm (72 hr).

Histopathological examination

Examination of control skin biopsies revealed the presence of mononuclear cells, one- to three-layers deep, around the small vessels within the papillary dermis. The histopathological pattern was similar for all the biopsies examined. At 6 hr after the injection of PPD, perivascular aggregates of lymphocytes, neutrophils and macrophages were present within the papillary dermis and small localized fibrin deposits could be seen subjacent to the epidermis. Leucocytoclastic vasculitis, involving most small blood vessels in the papillary dermis was apparent (Fig. 1a) and fibrin deposition within the walls of affected vessels was occasionally seen. Perivascular and perineural aggregates of cells similar to those found angiocentrally in the papillary dermis were present in the deeper layers of the reticular dermis. Between 12 hr and 24 hr after the injection of PPD, increased numbers of lymphocytes and neutrophils were distributed diffusely throughout the papillary dermis and leucocytoclastic vasculitis remained a marked feature of the reaction.

The intensity of the perivascular cellular reaction in the papillary dermis and the degree of fibrin deposition in the reticular dermis increased between 36 hr and 72 hr after the intradermal injection of PPD. However, leucocytoclastic vasculitis was no longer evident. The perivascular reaction was greatest at 72 hr and heavy perivascular cuffing with lymphocytes and macrophages was a consistent feature of the papillary dermis in sections from all cattle examined (Fig. 1b). The cellular infiltration consisted of lymphocytes and macrophages, and the neutrophils, many of which were necrotic, were confined to the secretory portions of the dermal sweat glands (Fig. 1c). Deposits of fibrin were present between the connective tissue stroma of the papillary dermis. The most striking feature of the reaction in the reticular dermis at 72 hr was the presence of interstitial inflammatory oedema associated with the deposition of fibrin around collagen bundles (Fig. 1d). Thrombosis of both lymphatics and capillaries was evident and perivascular and perineural aggregations of lymphocytes and macrophages were also present. Occasional giant cells of both the Langhans

and foreign body type were also observed within the reticular dermis.

Immunocytochemical identification of the infiltrating leucocytes

The mean number of leucocytes expressing the BoCD2, BoCD4, BoCD8, WC1, IL-2R and ACT2 antigens and the mean number of macrophages and neutrophils in control skin and at 6, 12, 24, 36, 48, 60 and 72 hr after the intradermal injection of PPD are shown in Fig 2. No B cells were detected in biopsies taken from either group of cattle throughout the period of study. No IgG was detected within the walls of blood vessels affected by leucocytoclastic vasculitis.

Necropsy findings

All the cattle in Group 2 were found on *post mortem* examination to have tuberculous lesions of focal lymphadenitis that were associated with calcium deposition and on occasion, obvious caseous necrosis. Lesions were located in the mediastinal and pulmonary lymph nodes in all the cattle. Histopathologically, tubercles, identified as areas of caseous necrosis surrounded by epithelioid macrophages and giant cells were seen in all the lesions examined. *M. bovis* was cultured from each lesion examined.

DISCUSSION

Gamma delta T cells were the most numerous T cells in the perivascular infiltrate between 6 and 12 hr after the intradermal injection of PPD. The precise function of $\gamma\delta$ T cells is uncertain. Antigen-specific $\gamma\delta$ T cells provide the initial immune response in mice infected with *M. tuberculosis*[16] and bacillus Calmétte–Guérin.[17] Gamma delta T cells also play an important role in the development of murine DTH reactions. The transfer of contact sensitivity by cells from immune mice required the presence of both $\alpha\beta$ T cells and a regulatory population of $\gamma\delta$ T cells.[18] Gamma delta T cells expressing the IL-4 receptor were essential for the transfer of DTH to trinitrophenyl and may induce the DTH reaction by allowing the extravasation of antigen-specific $\alpha\beta$ T cells at the site of antigen challenge.[19] Immunocytochemical examination of the control skin in the present study revealed the presence of a 'resident' perivascular population of $WC1^+$ $\gamma\delta$ T cells. Following the intradermal injection of PPD, cytokines released following activation of these 'resident' $\gamma\delta$ T cells may have induced expression of leucocyte adhesion molecules facilitating recruitment of antigen-specific $\alpha\beta$ T cells to the site of antigen challenge.

The murine tuberculin reaction is mediated by a population of antigen-specific $CD4^+$ cells following recognition of antigen processed by antigen-presenting cells in association with major histocompatibility complex (MHC) class II molecules.[20,21] Furthermore, immunocytochemical studies of the tuberculin reaction in man support the view that the human tuberculin reaction is also mediated by a population of $CD4^+$ cells.[2,3] The BoCD4 molecule, the bovine orthologue of CD4, is similar in molecular weight and tissue distribution to the human and murine equivalents and the antigen recognized by monoclonal antibody CACT138A in the present experiment is the bovine orthologue of CD4.[7,22] Bovine $CD4^+$ cells recognize antigen presented by macrophages in association with MHC class II molecules[7] and the infiltrating $BoCD4^+$ population probably represents the antigen-specific, effector population of T cells essential for the amplification of the bovine tuberculin reaction.

Examination of the tuberculin reaction at 72 hr after the intradermal injection of PPD revealed that most of the increase in skin thickness was because of inflammatory oedema within the reticular dermis (Fig. 1d). The fundamental importance of fibrin deposition to interpretation of the Mantoux test was demonstrated by studies that found that tuberculin reactions in human patients with congenital afibrinogenaemia lacked induration and swelling.[23] The mechanisms that contribute to inflammatory oedema and the deposition of fibrin in DTH reactions are inadequately understood. However, murine studies suggest that lymphokines released from activated T cells play an important role in the development of the inflammatory oedema.[24,25] Interferon-γ (IFN-γ) produced by a population of antigen-specific $CD4^+$ Th1 cells was responsible for over 50 per cent of the inflammatory oedema in the murine DTH reaction to KLH.[25] Interleukin-2 increases vascular permeability in mice[26] and Th1-derived IL-2 may also contribute to the inflammatory oedema in murine DTH.[25]

Increased inflammatory oedema in the present experiment occurred contemporaneously with an increase in the expression of the IL-2R. The expression of the IL-2R on the surface of bovine T cells develops following presentation of antigen by an antigen-presenting cell.[27] A marked increase in the number of infiltrating $BoCD4^+$ cells occurred between 48 hr and 72 hr when both the intensity of inflammatory oedema and expression of the IL-2R were maximal. High-density expression of the IL-2R has been demonstrated on activated $BoCD4^+$ cells[27] and production of cytokines by activated $BoCD4^+$ cells probably contributed to the development of the inflammatory oedema in the bovine tuberculin reaction. The mechanisms by which cytokines increase vascular permeability in DTH reactions are uncertain. However, they may have a direct effect on vascular permeability or an indirect effect through the release of free radicals and prostaglandins from macrophages.[26] Macrophages were the most numerous leucocytes found in the perivascular infiltrate between 12 hr and 72 hr after the intradermal injection of PPD. Macrophages are essential to the development of murine DTH reactions as they initiate the reaction by presenting antigen in association with MHC molecules to previously sensitized $CD4^+$ cells.[28,29] Activated macrophages are also important in the production of fibrin in DTH reactions by stimulating local activation of clotting mechanisms.[30]

Leucocytoclastic vasculitis has not previously been described in the tuberculin reaction of any species. In the present study, leucocytoclastic vasculitis was closely associated with the period of neutrophil and $\gamma\delta$ T-cell infiltration and the likely release of inflammatory mediators from necrotic neutrophils may have contributed to the vascular damage. Human $\gamma\delta$ T cells activated *in vitro* by *M. tuberculosis* exhibited cytolytic activity[31] and $\gamma\delta$ T-cell-derived cytokines may also have contributed to the pathogenesis of the vasculitis. The view that an Arthus reaction could be 'superimposed' on the tuberculin reaction was expressed by several authors.[32,33] Deposits of IgG were not detected within the walls of the inflamed blood vessels in the present study and no evidence was found to link leucocytoclastic vasculitis to a 'superimposed'

Arthus reaction. The absence of B cells from the reaction is consistent with immunocytochemical studies of the human tuberculin reaction[2,3] and with the view of the tuberculin reaction as a T-cell-mediated DTH response.

The molecule BoCD8 is the orthologue of human CD8 and equivalent to similar molecules in other species.[34] A population of BoCD8$^+$ cells exhibited MHC class I-restricted cytotoxicity[34] and activated BoCD8$^+$ cells may have contributed to the development of vascular damage and inflammatory oedema throughout the tuberculin reaction. There is also evidence that a population of BoCD8$^+$ $\gamma\delta$ T cells expressing the ACT2 antigen functions as suppressor cells.[14] The ACT2 antigen is expressed on all activated $\gamma\delta$ T cells, including a subpopulation of WC1$^-$ $\gamma\delta$ T cells that co-express BoCD8$^+$.[35] A population of ACT2$^+$ BoCD8$^+$ $\gamma\delta$ T cells may function as regulatory cells during the bovine tuberculin reaction.

The monoclonal antibodies used in the present study allowed only the phenotypic identification of subpopulations of bovine T cells involved in the tuberculin reaction. The precise function and the cytokine secretion pattern of the subpopulations of bovine T cells remains unknown. The *in situ* examination of the expression of cytokine mRNA and a study of the response to the intradermal injection of PPD in cattle that had not been sensitized to mycobacterial antigens could provide valuable information regarding the specific role of the various T cells involved in the reaction.

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