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Cyclosporin A abrogates the acquired immunity to cutaneous reinfection with the parapoxvirus orf virus

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SUMMARY

The effect of cyclosporin A (CsA) on host immunity to cutaneous reinfection with the parapoxvirus orf virus was studied in 6-month-old lambs. In control reinfected animals, clinical lesions and viral replication (measured by the presence of vesicular/pustular lesions and viral antigen) in regenerating epidermal cells were at a maximum on day 4 with resolution by day 9. Lesion histology revealed recruitment of T cells, B cells and dermal dendritic cells (DDC) which increased and decreased in parallel with the clinical course of the reinfection. In animals treated with CsA (25 mg/kg/day) 1 day before and for 8 days after reinfection, more severe clinical lesions and viral replication typical of primary infections were recorded and had not resolved by 28 days following reinfection. During CsA treatment, the recruitment of T cells, B cells and DDC was inhibited. With cessation of CsA treatment there was dramatic recruitment of $CD4^+$ T cells followed by DDC then B cells to the lesion site but rapid onset of acquired immunity was not recorded. Reverse transcription–polymerase chain reaction (RT–PCR) analysis of cytokine mRNAs from lesion biopsies showed individual sheep variations. However, interleukin-2 (IL-2) and interferon-γ (IFN-γ) mRNAs were detected in the control reinfected animals on days 3 and/or 9 after reinfection but not on these days in animals undergoing treatment with CsA. In the untreated lambs there was an inexplicable lack of IL-2 and IFN-γ mRNAs on day 6 after reinfection. Tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) mRNAs were unaffected by CsA treatment. The data suggest that CsA abrogates acquired immunity to orf virus reinfection by targetting T-cell lymphokine production.

INTRODUCTION

Orf is an acute debilitating skin condition of sheep and goats that also affects man.[1] The causative pathogen is orf virus, a DNA parapoxvirus that infects via broken or scarified skin and replicates in regenerating epidermal cells.[2] The virus can repeatedly reinfect skin sites on previously infected animals, albeit with a reduced lesion size and time to resolution compared to a primary infection. This is in spite of the build-up of an apparently normal T-cell and antibody response as measured either systemically or locally in afferent and efferent lymph.[3–6] The skin lesion is characterized by an early accumulation of neutrophils followed by $\gamma\delta$ $receptor^+$ T cells, $\alpha\beta$ $receptor^+$ T cells and B cells.[7,8] An unusual feature is the accumulation of a dense network of major histocompatability complex (MHC) class-II^+ dendritic cells underneath and adjacent to orf virus-infected epidermal cells.[9,10] This study sought to determine whether the acquired immunity to orf virus reinfection could be affected by the immunosuppressant drug cyclosporin A (CsA). In particular, we wished to determine whether treatment with CsA influenced: (a) the clinical severity of the disease; (b) the recruitment of immune and inflammatory cells to the site of reinfection; and (c) cytokine mRNA production by cells in the lesion.

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Abbreviations: CsA, cyclosporin A; CHO, chinese hamster ovary; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; IFN-γ, interferon-γ; KGF, keratinocyte growth factor; VEGF, vascular endothelial growth factor; TNF, tumour necrosis factor; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; RT–PCR, reverse transcription–polymerase chain reaction; ov, ovine; bov, bovine; rov, recombinant ovine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Animals and experimental procedure

To ensure simultaneous recent exposure to orf virus, Suffolk-cross lambs aged 6 months were infected with about 10^6 Moredun reference strain orf virus virions by scarification of the epidermis of the inner surface of the right hind leg.[2] Eight weeks later, fully recovered lambs were treated as follows:

Group A lambs ($n = 3$) were scarified on the left inner thigh and 0·1 ml phosphate-buffered saline (PBS) applied to the scratch line.

Group B lambs ($n = 3$) were reinfected, using 10^6 virus particles along the scratch line.

Group C lambs ($n = 3$) were reinfected and treated with CsA (25 mg/kg, Sandimmune, Sandoz Pharmaceuticals (UK) Ltd, Cambereley, Surrey, UK) via a jugular vein catheter daily for

9 days, starting 1 day before virus reinfection. The dose was determined by preliminary dose-response experiments, and the duration of treatment matched the time to clinical resolution of reinfection lesions in untreated sheep.[2–10] Group B lambs received vehicle only systemically.

Clinical scoring

The scarified areas of skin were examined daily for up to 16 days to assess formation of lesions as described previously.[11] The responses assessed were: erythema, vesicle and/or pustule and firmly attached scab. When present, each of these was scored depending on the width of the lesion along the scar line. A score of 1 was for a lesion <1 mm, 2 for a lesion 1–2 mm and 3 for a lesion >3 mm across. The total daily clinical score (TCS) for each sheep was the sum of the scores for the three types of lesion. The group mean score (GMS) was calculated by dividing the TCS values by the number of animals in each group.

Histological analysis of the skin

Two 6 mm punch biopsies were taken along the scar lines of lambs at 0, 3, 6, 9, 12 and 15 days following reinfection and on days 0, 3 and 6 in control group A lambs. At each time point, one specimen was taken immediately for RNA extraction. The other was fixed and processed for histology (haematoxylin and eosin staining), and immunohistology using an avidin–biotin peroxidase technique (Vectastain Elite, Vector Laboratories, Peterborough, UK) as described previously.[10] For quantification of cells three to five semi-serial paraffin wax sections of each sample were cut at intervals of at least 50 μm. Cryostat sections of fixed and frozen samples were required for the detection of $CD4^+$ and $CD8^+$ T cells. The following monoclonal antibodies (mAbs) were used: SBU-49.1, anti-ovine (ov) MHC class II;[12] 73B, anti-ovCD45RA, which in sheep skin stains B cells and <1% of T cells;[13] CC15, anti-ov/bovine (bov) $\gamma\delta$ T-cell receptor$^+$ (T19 antigen, WC1) T cells;[14] SBU-T4, anti-ovCD4 and SBU-T8, anti-ovCD8;[15] ILA-15, anti-bov/ov CD11b;[16] OM-1, anti-ovCD11c;[17] 2E5, specific for an orf virus envelope protein (P. F. Nettleton and H. W. Reid, unpublished). Isotype matched mAbs specific for border disease viral epitopes (G. Entrican, Moredun Research Institute) were used as controls. Dermal dendritic cells (DDC) were defined as mononuclear cells with prominent dendritic cytoplasmic processes which stained intensely for MHC Class II antigen and lacked the macrophage-associated antigens CD11b and CD11c[10] (as assessed by two-colour analysis or staining serial sections with mAb 49.1, and a cocktail of the mAbs OM-1 and ILA-15). Because of the dense accumulation of these cells in orf lesions, a semi-quantitative scoring system was devised as follows:

Score	DDC/mm^2 dermis
0	0–20
1	21–60
2	61–120
3	121–200
4	> 200

Other cell types were quantified in the dermis by counting four high power fields adjacent to and underneath infected epidermal cells in each section in three sections cut across the lesion. The results were presented as the number of cells per mm^2 of dermis.

Haematological and serological analysis

Total blood erythrocyte and leucocyte counts were performed on heparinized blood samples using a Coulter counter and standard technique. Differential cell analysis was performed on blood smears fixed and stained with Leishman solution. The blood plasma concentration of orf virus-specific antibody was measured using mAbs to orf virus proteins and a specific enzyme-linked immunosorbent assay (ELISA) as described previously.[3–5]

Reverse transcription–polymerase chain reaction (RT–PCR) for cytokine mRNA analysis

Skin biopsy specimens were diced and homogenized in guanidinium isothiocyanate and total RNA was prepared by acid phenol extraction. RNA was reverse transcribed and PCR was performed on the cDNAs as described previously.[18] To ensure the detection of amplified cDNA and not genomic DNA, primers were constructed from separate exons. The products were amplified in a DNA thermocycler (Hybaid Omnigene, Teddington, UK) for 20 and 35 cycles then electrophoresed on a 1% agarose gel, transferred onto a nylon membrane (Hybond M, Amersham, UK), probed with ^{32}P-labelled cytokine-specific oligonucleotides recognizing internal sequences followed by autoradiography. Biopsy glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a control. The relative intensity of the Southern blot autoradiograph bands for the different cytokines were scored as described previously[19] but based upon transmission light densitometry scanning. A score of 0 was for no detectable band, and 4 for a maximum intensity band.

RT–PCR controls included: specific cytokine cDNA amplification (all cytokines) and dilutions of mRNA from Chinese hamster ovary (CHO) cells expressing recombinant ovine (rov)IL-2 (unpublished), rovIL-3,[18] rovIFN-γ[20] and rov granulocyte–macrophage colony-stimulating factor (GM-CSF)[18] in a constant concentration of total mRNA from untransfected CHO cells and subjected to the RT–PCR. This controls for the reverse transcription as well as the PCR stages. PCR conditions were within the linear range of amplification when using 20 or 35 cycles. Negative controls consisted of PCR buffers and reverse osmosis-purified water, which gave no amplification products.

Ovine cytokine-specific primer sequences (5′ then 3′) were: IL-1β, (GAAGTGATGGCTTACTACAG and GTTATATCCTGGCCACCTCT; IL-2, ATGTACAAGATACAACTC and GTCATTGTTGAGTAGATG; IL-3, CGAAGGACCTGGACAAGACA and GTTGTCACTGATGATCTGAG; GM-CSF, AGTCCTCAAGAGGATGTGGC and GCGATCTGTGAGGTAAGCTT; IFN-γ, CATAACACAGGAGCTACCGA and TGCCAAGTTGGACCCTGAGA; TNF-α TTGCAGGAGCCACCACGCTC and CAGGTTGATCTCAGCACTGA; keratinocyte growth factor (KGF), TTATGTTATTCATGAACACC and TTAAGTTATTGCCATAGGAA; VEGF, GCCTCCGAAACCATGAACTT and TCTGGTTCCCGAACCCTGAG.

The sequences of the cytokine-specific oligonucleotide probes were: IL-1β, CGATGAGCTTCTGTGTGATGCAGCCGTGCAGTCAGTAAAA; IL-2, AGTTAAATGTATGCATCCTGGAGAGCTTGAGGTTCTCGGG; IL-3, TGGACTTCAGATAGTCTCTGCTGCTGCTAAGTCGTGTCTG; GM-CSF, CTTCACTTCTGGACTGGTTCCCAGCAGTCAAAGGGAATGA; IFN-γ GAAGTCCTCCAGTTTCTCAGAGCTGCCGTTCAAGAACTTC; TNF-α TTGATGGCAGAGAGGATGTTGACCTTGGTCTGGTAGGAGA; KGF, ATGGAAGGAGGAGATATAAGAGTGAGAAGACTCTTCTGTC; VEGF, GTGAAGTTCATGGATGTCTACCAGCGCAGCTTCTGCCGTC.

Full DNA sequences have been published[23] (and European Molecular Biology Laboratory accession number Z46236 for ovKGF).

Statistical analysis

Where appropriate, using normalized data (geometric means) and Student's *t*-test.

RESULTS

Clinical analysis

Scarification without reinfection (group A) caused a mild erythema which resolved within three days. Untreated group B lambs developed typical reinfection lesions which progressed through the clinical stages of erythematous macule, vesicle/pustule and scab formation with macroscopic resolution in all cases by day 9 (Fig. 1). The clinical progression, lesion size and time to resolve were similar in all lambs. Lesion size was at a maximum on day 4. In CsA-treated group C lambs, lesion development was similar up to day 4 following reinfection but developed further from day 5 to day 16 (Fig. 1), becoming severe and had not resolved by day 28 when the experiment was concluded. The clinical progression as well as lesion sizes were similar in each of these lambs.

Histological analysis

In group A, a small (< 25 cells/mm^2 of dermis) increase in neutrophils, lymphocytes and MHC class II$^+$ DDC was recorded in the papillary dermis on days 1–3 following scarification. In the control reinfected lambs (group B) there was progressive epidermal hyperplasia and thickening under the damaged and infected epidermis from day 3 till day 6 after reinfection when staining for orf virus antigen was at its most intense. Resolution, as measured histologically, had occurred by day 15. An accumulation of polymorphonuclear cells into the papillary dermis on day 1 was followed by a mononuclear cell infiltration consisting of MHC class II$^+$ DDC, CD4$^+$, CD8$^+$, T-19$^+$ T cells and CD45RA$^+$ B cells which reached a maximum on day 6 and declined to preinfection levels by day 15 (Figs 2, 3).

In the CsA-treated group C animals, epidermal thickening and hyperplasia as well as orf virus antigen staining was qualitatively similar to group B lambs over the first 6 days. During this time, which coincided with the daily treatment of CsA, the accumulation of all the different immune and inflammatory cells was less than that seen in the group B lambs (Fig. 2b and 2a compares MHC class II$^+$ DDC). On day 6, there were significantly fewer ($P < 0{\cdot}01$) dermal CD4$^+$ and CD8$^+$ T cells, B cells and DDC but not T19$^+$ $\gamma\delta$ T cells than in group B lambs (Fig. 3). Following the completion of CsA treatment of group C on day 8, there was evidence of continuing severe epidermal damage and a strong intensity of staining for orf virus antigen on days 9, 12 and 15. Underneath the damaged cells, marked epidermal thickening and hyperplasia proceeded to such an extent that rete formation (epidermal plugs penetrating the dermis), typical of primary lesions were widespread. Figure 3 shows that there was a marked increase in the numbers of CD4$^+$ T cells on days 9, 12 and 15, DDC on days 12 and 15 and B cells on day 15 compared to numbers recorded during the period of CsA treatment (also compare Fig. 2d to Fig. 2b). The numbers of these cells had not declined on day 15, the last day of histological analysis, and by which time the lesions in group B animals had resolved fully (Fig. 2c).

Haematological analysis

The blood leucocyte and erythrocyte counts as well as differential cell counts were within normal ranges for uninfected age matched controls (including group A animals) at all time points studied in the groups of lambs. The differences between group C and group B animals was not significant ($P > 0{\cdot}05$) for any cell-type comparison.

Antibody analysis

During the period of treatment with CsA there was a lower plasma antibody concentration in group C on day 7 ($P < 0{\cdot}04$) compared to group B (Fig. 4). After cessation of CsA treatment plasma antibody titres increased steadily in group C animals for the remaining period of analysis.

Cytokine mRNA analysis

Figure 5 shows the RT–PCR analysis of cytokine mRNAs in the lesions of one group B and one group C animal and test controls to validate the RT–PCR. Table 1 shows orf lesion mRNA scores for all the animals. There were variations in cytokine mRNA expression between the different animals in each group. However, the CsA-treated animals (group C) lacked detectable IL-2 and IFN-γ mRNA on days 3, 6 and 9 during and immediately after the period of CsA treatment. Following cessation of CsA treatment, transcription of these genes was detected on days 12 and/or 15. In group B, IL-2 and/or IFN-γ mRNAs were detected on days 3 and 9, in each case in two animals. IL-3 mRNA was less affected by CsA treatment than that of IL-2 and IFN-γ. TNF-α and VEGF mRNAs were unaffected by CsA treatment. GM-CSF, IL-1β and KGF mRNAs were detected only in a few lesion samples from the lambs of both groups.

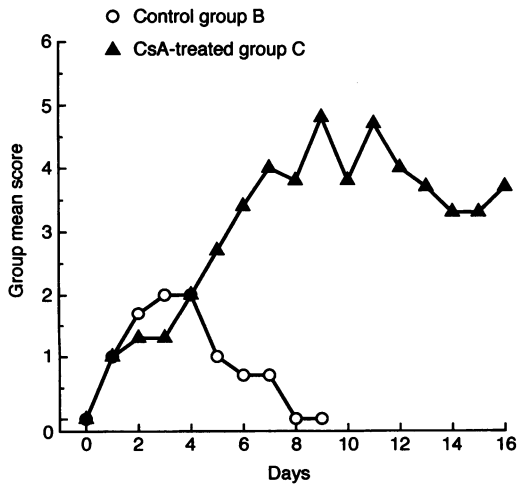

Figure 1. Clinical progression of orf lesions in CsA-treated (group C)and control reinfection lambs (group B). Clinical progression was erythema, vesicle/pustule then scab. The lesions were scored as described in materials and methods. The group mean score was derived from the total daily clinical score for each sheep which in turn was derived from a sum of the scores for the three types of lesion (erythema, vesicle/pustule and attached scab).

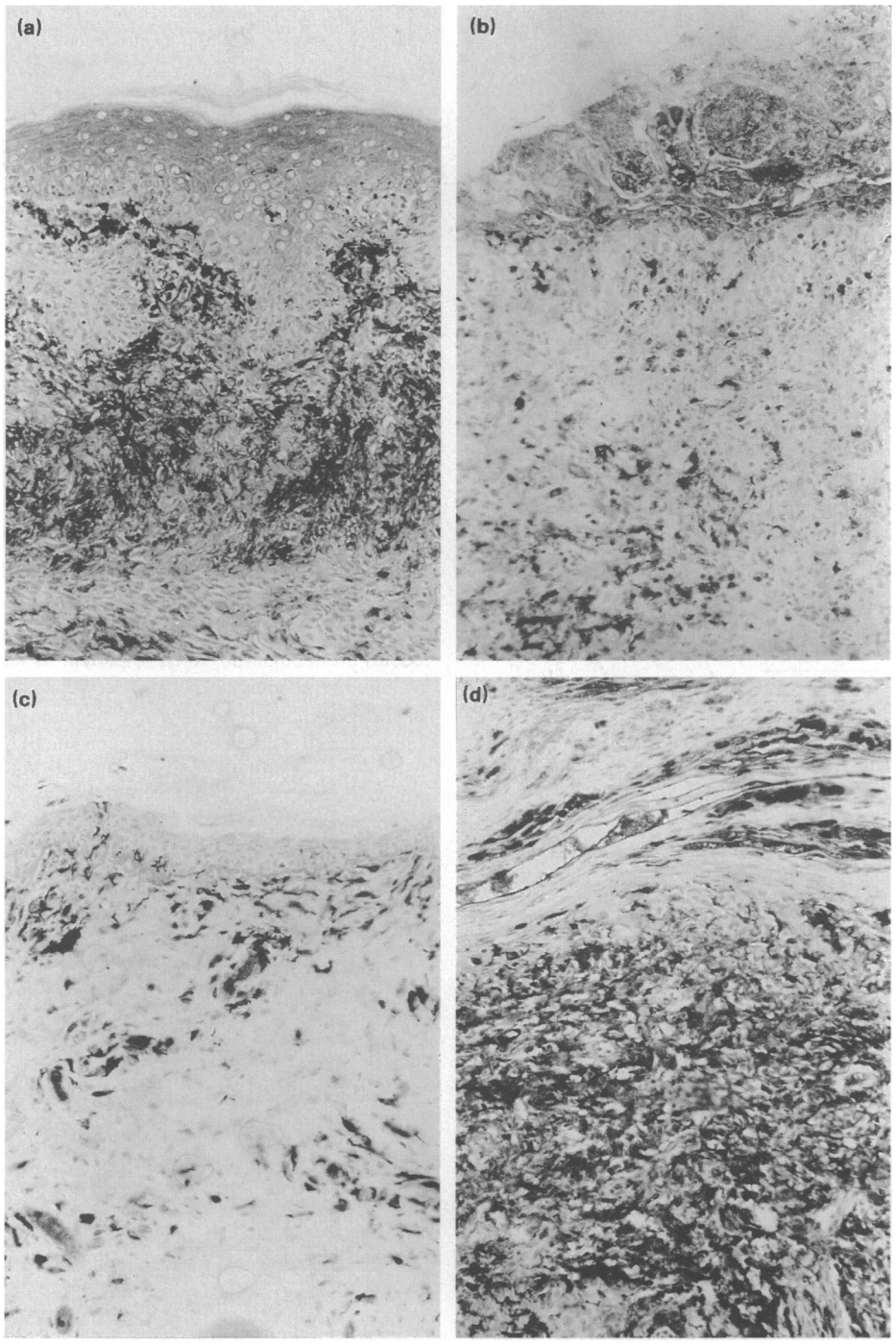

Figure 2. Orf lesion immunohistology. biopsy sections (10 μm) were stained with mAb 49.1 specific for ovMHC-class II antigens (originally dark brown) and mAb 2E5 specific for an orf virus envelope protein (originally red) using the peroxidase technique. The 49.1 antibody stains DDC and (with less intensity) B cells and activated T cells. DDC predominate in the sections shown. The 2E5 antibody stains orf virus in the degenerating epidermis, most notable in (a) and (b) and lacking in (c). (a) Lesion, day 6 following reinfection of group B lambs.(b) Lesion, day 6 following reinfection of group C (CsA-treated) lambs. (c) Lesion, day 15 following reinfection of group B lambs. (d) Lesion, day 15 following reinfection of group C lambs. All photographs $\times 87$.

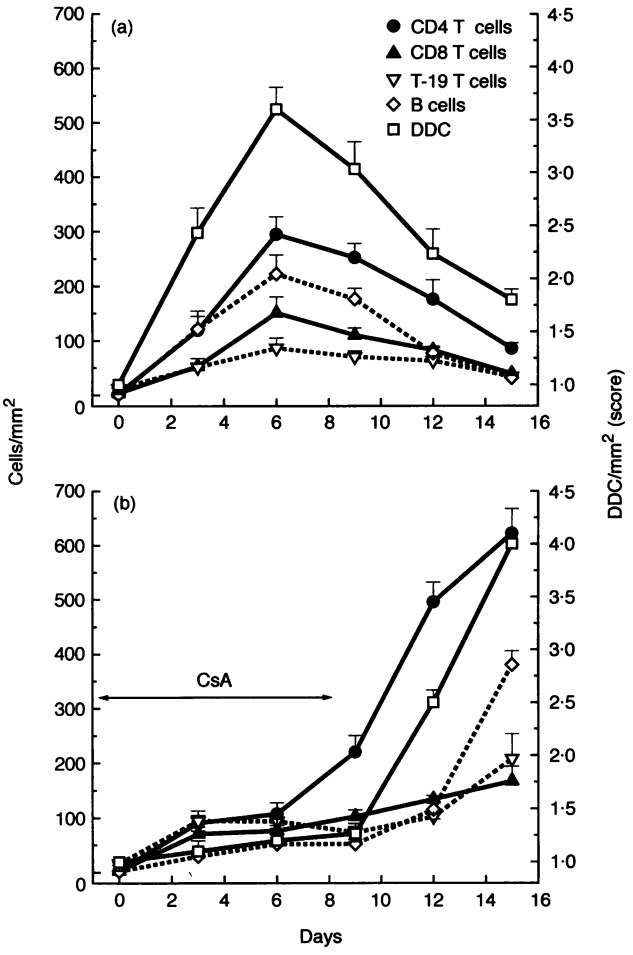

Figure 3. Recruitment of lymphocytes and dermal dendritic cells to the orf lesion. A time course study. Orf lesion biopsy sections stained with mAbs specific for the different cell types using the immunoperoxidase technique and quantified as described in materials and methods. (a) Control reinfection group B. (b) CsA treatment and reinfection group C.

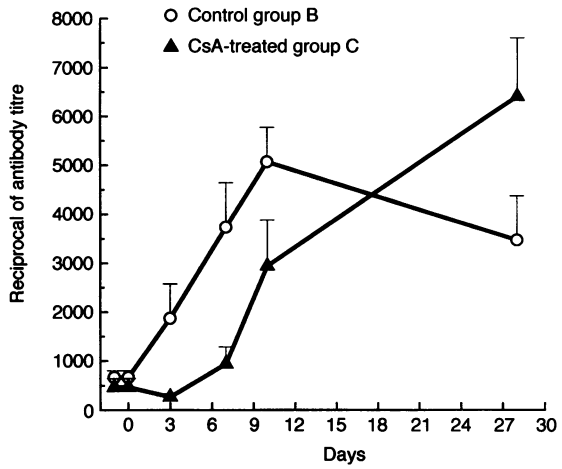

Figure 4. Orf virus-specific antibody in blood plasma from group B and group C (CsA-treated) lambs following reinfection. Antibody titres were measured by specific ELISA as described in materials and methods.

DISCUSSION

Lambs repeatedly infected with orf virus show evidence of acquired immunity as lesion size and time to resolution decrease with each exposure to the virus. However, this immunity is effective only after a delay during which there is viral replication and subsequent shedding of virus with the scab. The ability of the virus to replicate has hindered the development of an effective vaccine. In this study, the administration of 25 mg/kg/day of CsA systemically to lambs from 1 day before reinfection and for 8 days afterwards temporarily abrogated the development of acquired immunity (as measured clinically and histologically). As far as we are aware, this is the first demonstration of CsA treatment of a poxvirus-infected mammalian host.

CsA did not affect the initial phase of lesion development up to 4 days after reinfection, but thereafter allowed a level of viral replication (intensity of staining of orf virus antigen)[2,7,10] and lesion development that was indistinguishable from events in primary lesions.[2,7,10] The virus (as antigen) remained associated with replicating epidermal cells and showed no evidence of systemic spread. Previous studies have shown an apparently normal primary and anamnestic skin and/or lymph immune response to virus infection involving activated total and antigen-specific $CD4^+$ and $CD8^+$ T cells, B cells and specific antibodies.[4–10,22,23] Antibodies are thought not to be important as high titres of passively acquired antibodies do not protect young lambs against primary challenge with orf virus.[24] Cell-mediated immunity is therefore implicated. Recruitment to the lesion site of $CD4^+$ T cells, $CD8^+$ T cells, B cells and DDC was suppressed in the CsA-treated lambs during the period of treatment. However, with cessation of CsA treatment dramatic recruitment of $CD4^+$ T cells, followed by DDC then B cells to the site of reinfection took place. $\gamma\delta$ T cells and $CD8^+$ T cells had not accumulated to the same extent on day 15, the last day of histological analysis. In spite of the recruitment of these cells, the amount of virus continued to increase at least until day 15 (as measured histologically) and lesions persisted until at least 28 days following reinfection. A rapid onset of acquired immunity after cessation of CsA treatment therefore was not seen. The pronounced recruitment of the different cells 1–2 days following termination of the CsA treatment would argue against a residual effect of CsA in the tissues on the delayed acquisition of immunity.

Messenger RNA levels for IFN-γ and IL-2, which are associated with activated T cells and natural killer (NK) cells, were not detected during the period of CsA administration to the lambs, in spite of the presence of virus antigen. This is consistent with an immunosuppressive function of CsA on IL-2 and IFN-γ gene transcription.[25] T-cell numbers in the lesions of CsA-treated animals and their controls were not markedly different on days 3 and 9 when control animals showed an IL-2 and/or IFN-γ mRNA response and CsA-treated animals did not. There was an inexplicable lack of IL-2 and IFN-γ mRNAs in the lesions of reinfected control lambs on day 6 when T cells were at their most numerous. In support of a preferential action of CsA on T cells, TNF-α mRNA, which is produced by a wide range of nucleated cells, was not inhibited by CsA. KGF and VEGF were included in the study as they are implicated in epidermal cell proliferation and angiogenesis, respectively, both of which are present in orf lesions. Messenger RNAs for these cytokines were detected after reinfection of both groups of sheep. VEGF mRNA, which is thought not to be produced by T cells, was not affected by CsA treatment. The results suggest that the effect of CsA is principally

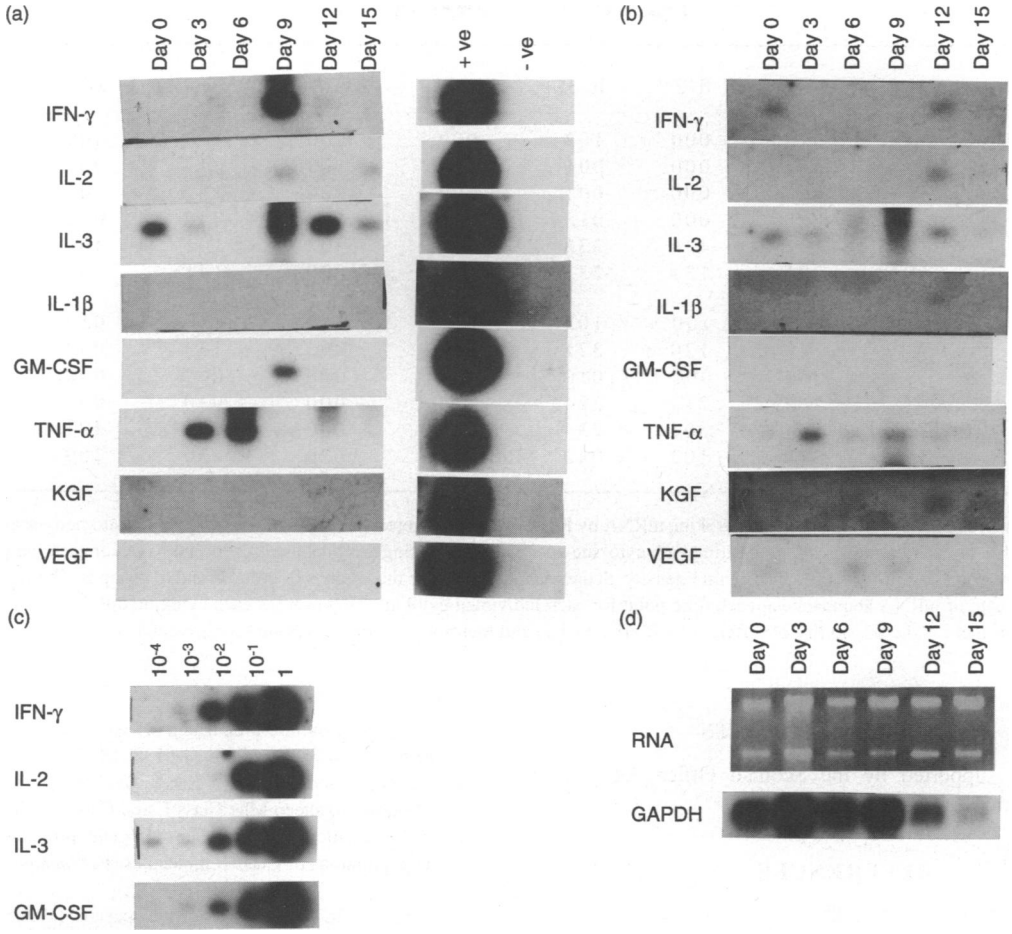

Figure 5. RT–PCR analysis of cytokine mRNAs in orf lesion biopsies. (a) RNA samples from orf lesions taken following reinfection of a group B lamb were subjected to RT–PCR as described in materials and methods. Amplified cytokine-specific cDNA was run on a 1% agarose gel, blotted onto nylon membranes and subjected to Southern analysis using cytokine-specific ^{32}P-labelled oligonucleotide probes and then exposed to X-ray film. Positive controls (+ve) are RT–PCR performed on cDNAs for the different cytokines. Negative controls (−ve) are RT–PCR performed on water used to prepare the assay buffers. (b) RT–PCR analysis of cytokine mRNAs from a CsA-treated group C lamb. Note the lack of IL-2 and IFN-γ mRNAs on days 3, 6 and 9 after reinfection. (c) Dilutions of mRNA from CHO cells expressing recombinant ovine cytokines in a fixed total concentration of untransfected CHO mRNA were used to prove the RT–PCR technique. (d) Total RNA and specific GAPDH mRNA for each of the lesion samples loaded on the gel using a fixed concentration of total RNA. Equalization of the GAPDH signal (not shown) did not affect the differences in lesional score analysis between the groups (shown in Table 1), in spite of an alteration in score for some samples.

on IL-2 and IFN-γ production and that these lymphokines are required for acquired immunity to orf virus infection. This, together with the result that CD4$^+$ T cells were recruited to the lesion more rapidly than the other cells following the removal of CsA treatment, suggests that activated CD4$^+$ T cells may be required (at least in part) for the recruitment of the DDC which are a marked feature of the orf lesion.

The ability of orf virus to evade host immunity, at least temporarily, in reinfections has not been explained. One possibility is that orf virus, in common with other poxviruses[26] can subvert or block mediators of protective immunity for a short period of time. Orf virus virulence genes with homologies to mammalian immune and inflammatory response genes have been discovered,[27,28] including a putative interferon resistance gene and a homologue of VEGF. If such virulence mechanisms were active, a situation could be envisaged in which, following reinfection, memory T cells are recruited and activated rapidly so that viral replication and the production of viral anti-host immunity-interference factors are limited to only a short period of time as the balance is in favour of the T cells. In contrast, in lambs treated with CsA, the balance is in favour of the virus which replicates uninhibited by an immune response during the later stage of CsA treatment. T cells recruited following termination of the CsA treatment, would be overwhelmed until they were numerous enough to counteract the putative viral mediators of escape from protective immunity.

The lower plasma concentrations of orf virus-specific antibodies in the CsA-treated lambs 7 days following reinfection may be due to reduced CD4$^+$ T-cell help for B-cell differentiation and antibody production, although a direct effect of CsA on B cells or plasma cells cannot be ruled out.

Finally, this study demonstrates that the tissue damage in orf is due primarily to the action of the virus replicating in regenerating epidermal cells rather than to the action of the host immune and inflammatory response to the virus.

Table 1. Orf lesion cytokine mRNA scores

Group	Day	IFN-γ	IL-2	IL-3	GM-CSF	IL-1β	TNF-α	KGF	VEGF
C	0	012	000	102	000	000	110	000	110
(CsA	3	000	000	001	000	000	313	000	111
treated)	6	000	000	001	000	000	131	001	343
	9	000	000	013	020	000	332	001	141
	12	402	434	332	000	402	101	404	000
	15	231	131	231	200	000	141	101	000
B	0	000	100	103	100	000	010	010	010
(vehicle	3	210	120	321	000	000	133	030	020
only)	6	000	000	010	000	000	044	000	340
	9	124	212	224	104	000	030	000	320
	12	031	301	234	000	000	122	401	200
	15	200	102	042	000	020	030	200	000

Biopsy samples were analysed for cytokine mRNA by RT-PCR. Scores were based on band intensity by densitometry scanning of autoradiographs following southern blotting with cytokine-specific probes, using an equal loading of total RNA for each sample. 0–4 represents quadrants of 0–100% maximum intensity. Scores are shown for the three lambs of group C and of group B. The scores give an estimate of mRNA abundance at each time point for each individual cytokine. However, as each cytokine mRNA is intrinsically different there is the possibility of different PCR efficiencies, and therefore the inter-cytokine scores cannot be compared.

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