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Cytokine gene expression in skin of susceptible guinea-pig infected with *Treponema pallidum*

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SUMMARY

Using a semi-quantitative multiplex reverse transcription–polymerase chain reaction assay, we examined cytokine mRNA expression for interleukin-1α (IL-1α), IL-2, IL-10, IL-12p40, tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) in skin samples obtained from C4-deficient (C4D) guinea-pigs inoculated intradermally with virulent *Treponema pallidum* (VTP). Controls included unmanipulated animals, guinea-pigs injected with *T. pallidum*-free rabbit inflammatory testicular fluid (ITF) alone, or mixed with heat-killed organisms (HKTP). The expression of IL-1α, IL-12p40, and TNF-α mRNA [T helper type 1 (Th1)] remained within the normal range in both infected and control animals throughout the experimental period. However, a significant increase ($P<0{\cdot}05$) in IL-10 mRNA (Th2) was found exclusively in the VTP-inoculated animals from 3 to 30 days post-infection. Another unique characteristic of the inflammatory response in infected guinea-pigs was the appearance, between 11 and 30 days post-inoculation, of a substantial number of eosinophils in addition to infiltrating mononuclear cells. The results showed a local Th2 response which is consistent with an inadequate immune response. This is reflected by the lengthy and incomplete clearance of the pathogen from the local site of entry and the chronic infection of distant organs.

INTRODUCTION

It is known that resistance to reinfection with virulent *Treponema pallidum* (VTP) is a T-cell mediated process.[1,2] Also known is the fact that acquisition of immunity in syphilis is a lengthy and deficient process.[2,3] The pathogen is not fully eradicated, allowing the occurrence of secondary and tertiary stages of reactivation in a substantial number of untreated individuals.[3] The local and systemic persistence of the pathogen far beyond the healing of local lesions and establishment of 'chancre immunity' has been reported by several investigators[4,5] and confirmed in the rabbit[6] and guinea-pig models of syphilis (submitted for publication). We have shown that the guinea-pig is a suitable host for studies of experimental syphilis,[7,8] and delineation of mechanisms of immunity.[2] There are several strains of guinea-pig displaying different susceptibilities to cutaneous inoculation with VTP. The Albany (Alb), a subline of Hartley strain is resistant [median infective dose (ID_{50}) $>10^9$ organisms], whereas the C4-deficient (C4D) strain is highly susceptible ($ID_{50}=10^2$ organisms).[8] Although the incidence of lesions in inbred strains 2 and 13 is lower compared to that of C4D (85% versus 100%), neither the character of the lesions nor the humoral response in the three strains differ significantly.[8]

To gain further insight into the local mechanism of immune response facilitating local persistence and systemic dissemination of the pathogen, we selected the C4D animals to examine the effect of VTP infection on the cutaneous expression of mRNA for six cytokines interleukin-1α (IL-1α), IL-2, IL-10, IL-12p40, tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) representing T helper type 1 (Th1) and type 2 (Th2) immune responses.

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MATERIALS AND METHODS

Animals

Adults (3- to 5-month-old) C4D guinea-pigs genetically related to inbred strain 13[8] were chosen for this study owing to their consistent (100%) cutaneous response to VTP. The C4D guinea-pigs have a genetically controlled total deficiency of the fourth component of complement,[9] however, their immunological competence at the cellular and humoral levels is similar to that of complement-sufficient strains.[10,11] These animals originated from the National Institutes of Health (Bethesda, MD) and were reared in our institution since 1975.

Preparation of treponemal suspensions and inoculation

Adult New York State Flemish Giant (NysFG) rabbits were inoculated intratesticularly with $\approx 10^7$ *T. pallidum* Nichols

strain. At the peak of orchitis (9–14 days), the animals were killed and the testes were aseptically removed. The testes were minced and swirled for 20 min in RPMI-1640 medium. The preparation was centrifuged for 10 min at 1200 r.p.m. to remove cells and tissue debris. The supernatant containing most of the virulent *T. pallidum* with some inflammatory testicular fluid was designated (VTP/ITF) and adjusted to contain 10^8 organisms/ml. A portion of the latter suspension was further centrifuged for 60 min at 30 000 r.p.m., and the supernatant was passed through a 0·2-μm filter. This treponema-free supernatant was designated ITF. The sediment containing the *T. pallidum* was resuspended in phosphate-buffered saline and then placed in a water-bath at 56° for 2 hr to kill the organisms (HKTP). The dead organisms were washed twice, and adjusted to the original concentration (10^8 organisms/ml) with ITF (HKTP/ITF). Intratesticular inoculation of ITF and HKTP/ITF into normal rabbits did not cause inflammation or seroconversion for a period of 3 months.

C4D guinea-pigs were intradermally infected at a single site in each marked and shaved hind leg with 0·1 ml (10^7) of freshly prepared VTP/ITF. Controls included unmanipulated animals and those injected at the same sites with a similar number of HKTP/ITF or the equivalent volume of ITF alone.

Collection of samples

At various times after inoculation the animals were killed by an intravenous injection of 1 ml of Sleepaway (Fort Dodge Laboratories Inc. Fort Dodge, IA). Both hind legs of experimental and control guinea-pigs were closely clipped and thoroughly disinfected. To prevent cross-contamination, individual sets of sterile blades and forceps were used to dissect $\approx 10\ mm^2$ of the inoculated skin site in both hind legs. Similar specimens were collected from the unmanipulated animals. Specimens obtained from animals representing the various groups were also prepared for histological examination. In the infected animals, care was taken to collect the skin lesions at the peak of development just prior to ulceration. Specimens were processed immediately for RNA extraction. Total RNA was immediately isolated using Ultraspec™ (Biotecx, Houston, TX). The integrity of the RNA sample was verified by agarose gel electrophoresis after denaturation with glyoxal and dimethyl sulphoxide[12,13] and storage at −80°.

Animal procedures have been approved by the Institutional Animal Care and Use Committee of the Wadsworth Center for Laboratories and Research.

Guinea-pig cytokine cDNA

We have generated full-length or partial cDNA clones for several guinea-pig cytokines including IL-2, IL-10, IL-12p40, TGF-β, IL-1α and TNF-α. The cloning and sequencing of these clones have been reported.[14] Two strategies were used to generate the cDNA clones. Two guinea-pig sequences (IL-1α, and TNF-α) were already in the GenEMBL database.[15] We synthesized appropriate primers and constructed partial cDNA using standard reverse transcription–polymerase chain reaction (RT-PCR) methodologies.[12] Guinea-pig sequences for the remaining cytokines (IL-2, IL-10, IL-12p40 and TGF-β) had not been previously reported. To generate these cDNA, cytokine sequences (human, mouse, bovine, rat, monkey) in the GenEMBL database were analysed using the PILEUP program in the GCG software package.[16] Highly

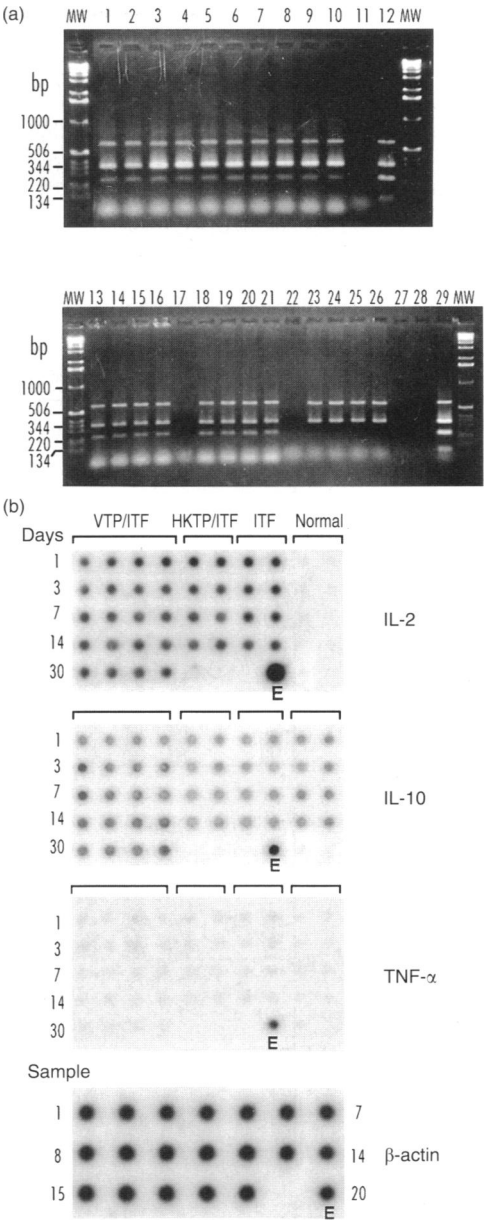

Figure 1. Analysis of cytokine gene expression in skin by RT-PCR and dot blot analysis. (a) Multiplex PCR analysis, amplified cDNA for β-actin (592 base pairs; bp), IL-10 (335 bp), IL-2 (235 bp), and TNF-α (105 bp) are shown. Lanes 1–10 represent VTP/ITF-injected guinea-pigs analysed in groups of two at 1 (lanes 1 and 2), 3 (lanes 3 and 4), 7 (lanes 5 and 6), 14 (lanes 7 and 8) and 30 days (lanes 9 and 10) of infection. Individual guinea-pigs injected with HKTP/ITF (lanes 13–16), and ITF (lanes 18–21) at 1, 3, 7 and 14 days of injection, respectively. Other lanes contain: four unmanipulated controls (lanes 23–26), a positive control, LPS-treated guinea-pig peripheral blood (lanes 12, 29), negative controls, H_2O (lanes 11, 17, 22), irrelevant DNA (lane 27) and no reverse transcriptase (lane 28). (b) Dot blot analysis, duplicate samples from animals killed at various times after inoculation with VTP/ITF (two guinea-pigs), HKTP/ITF (one guinea-pig), ITF (one guinea-pig). Four normal controls were killed at once. A single sample from each were also analysed for β-actin. A positive control (E), LPS-stimulated peripheral blood lymphocytes, was also included. The stronger expression of IL-10 mRNA in infected animals compared to controls is evident in (a) and (b).

conserved sequences spanning at least one intron were identified and primers with or without restriction sites were synthesized. Total RNA, isolated from concanavalin A (Con A)-stimulated guinea-pig spleen cells were reversed transcribed using random hexamers. Cytokine cDNA were then amplified by PCR using appropriate primers. Amplified products that had restriction sites were cloned into pBluescript® (Stratagene, La Jolla, CA) using standard methodologies. Amplified products lacking restriction sites were cloned directly into the TA cloning vector® (Invitrogen, San Diego, CA). Two clones from each of three independent cloning experiments were sequenced twice in both the forward and reversed directions. The sequence data is reported in Scarozza *et al.*[14] The sequence of the house-keeping gene β-actin is highly conserved; the primers used for its detection in guinea-pig were those reported by Rottman *et al.*[17]

Semi-quantitative multiplex RT-PCR analysis

Cytokine gene expression in skin specimens collected from infected and control animals was analysed using a semi-quantitative multiplex PCR.[18] For the multiplex PCR, we had to establish the conditions that resulted in exponential

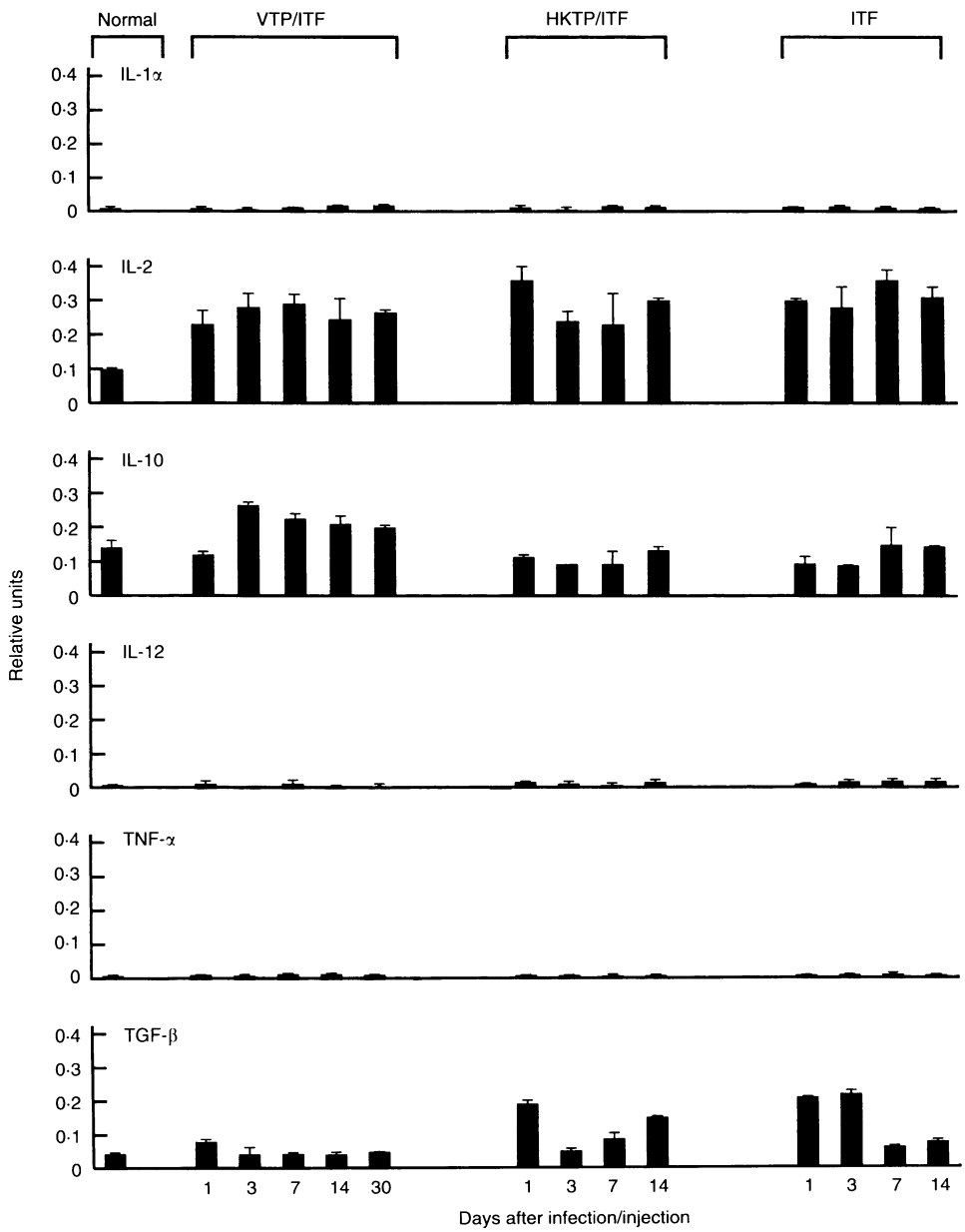

Figure 2. Kinetics of cytokine gene expression for IL-1α, IL-2, IL-10, IL-12p40, TNF-α and TGF-β in skin of infected and control guinea-pigs. The amplified cDNA was analysed by dot blot hybridization, quantified using a phosphoimager, and normalized to β-actin cDNA. The values were expressed as relative units (RU). Each bar represents the mean of each group (four normals, three infected and three of each manipulated controls) killed between 1 and 30 days of treatment. Spontaneous background levels for each cytokine are represented by the group of normal guinea-pigs. Statistically significant differences ($P<0{\cdot}05$) were noticed only for IL-10 expression between infected (RU = 0·20 0·28) and manipulated or unmanipulated control (RU ≤ 0·14) animals.

amplification of each sequence. Our PCR conditions consisted of 32 cycles of amplification: 94° for 30 seconds; 53° for 30 seconds; 65° for 4 min. Three cytokine sequences and an internal control (β-actin) were amplified simultaneously. Amplified cDNA were analysed by gel electrophoresis and quantified by dot blot hybridization.[14] Briefly, DNA samples were denatured in 0·3 M NaOH and applied onto each of four nylon membrane filters using a HYBRI-DOT manifold (Life Technologies Inc. Gaithersburg, MD). Each filter was probed separately with the appropriate cDNA sequence that had been radiolabelled with ^{32}P by random priming. Each filter was hybridized overnight to one of the four ^{32}P-labelled cDNA probes. Filters were washed under high stringency conditions [0·1 × saline–sodium citrate buffer (SSC), 0·1% sodium dodecyl sulphate at 65°]. Hybridization signals were quantified using a Phosphoimager (Molecular Dynamics, Sunnyvale, CA). Cytokine gene expression is reported in relative units (RU) as described in the Results. Results were statistically analysed using the Fisher's exact test and two-tailed *P*-values.

RESULTS

Course of infection

In the infected animals, lesions began to develop at ≈6–7 days, evolving into dark-field-positive ulcerative lesions, at ≈10–14 days after infection. They become totally healed in ≈30–45 days. The clinical course of syphilis has been described.[7] No lesions developed in control animals.

Cytokine gene expression

We initially performed a kinetic study of cytokine expression. Skin samples were collected from 43 adult C4D guinea-pigs (15 inoculated with VTP/ITF, 12 with HKTP/ITF, 12 with ITF, and 4 unmanipulated). Experimental and control animals were killed in groups of three at 1, 3, 7, 14 and 30 days after infection or at 1, 3, 7 and 14 days following injection with HKTP or ITF. The four unmanipulated animals were killed at once. A representative multiplex PCR and dot blot analysis of skin specimens obtained from infected, and controls guinea-pigs are shown in Fig. 1(a) and (b), respectively, and the quantitative results of the 43 animals are shown in Fig. 2. Cytokine gene expression is reported in RU, which is the ratio of the hybridization signal of amplified cytokine cDNA to that of amplified β-actin cDNA ± standard error of the mean (SEM). Each bar represents the mean value of three or four animals. While negligible levels (RU ≤ 0·01) of IL-1α, IL-12p40 and TNF-α (Th1) were found in both experimental and control animals, IL-10 mRNA (Th2) expression was significantly ($P < 0·05$) increased (RU = 0·20–0·28) over control levels (RU ≤ 0·14) from day 3–30 after infection. In infected animals IL-2 levels were substantially increased (RU = 0·23–0·29) in relation to normal animals (RU = 0·01), but were similar to or below those of sham controls (RU = 0·24–0·37). TGF-β mRNA was slightly increased (RU = 0·08) after one day of infection with VTP/ITF, but then declined to normal (RU = 0·05), while control values were substantially higher at day 1 but fluctuated thereafter. These results were further confirmed in a second set of experiments including four normal guinea-pigs, four infected, three injected with HKTP/IFT, and three injected with IFT (Fig. 3). These animals were killed at ≈14 days when the peak of lesion development has been reached in the infected guinea-pigs. In these experiments, analysis of TGF-β was not included.

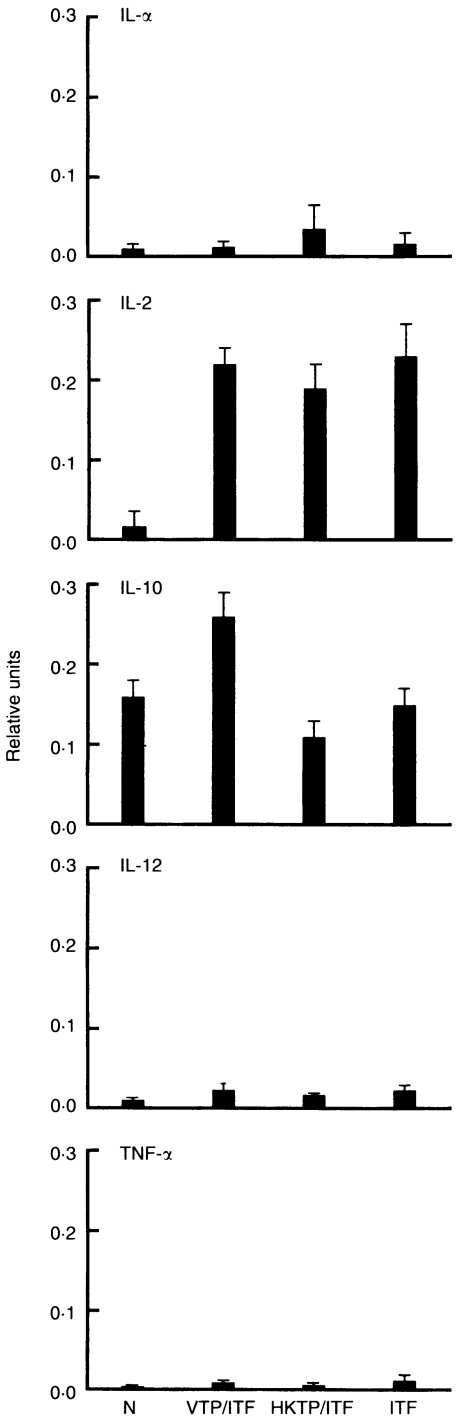

Figure 3. Cytokine gene expression in skin of infected and control animals 14 days after treatment. Tissues collected from four normals (N), four infected (VTP/ITF), three HKTP/ITF-injected and three ITF-injected animals were analysed using the semi-quantitative RT-PCR. TGF-β was not examined in these animals. Results are expressed in RU ± SEM. Significant differences ($P < 0·05$) were noticed only for IL-10 between infected and unmanipulated or manipulated control groups.

Histological findings

Histological examination of inoculated skin samples indicated two different patterns of local response in experimental and control animals (Fig. 4). A temporary, very mild scattered infiltration of mononuclear cells developed in control animals from 1 to 3 days after injection of HKTP/ITF or ITF. On the other hand, an increasing hypertrophy of the epidermis, a dermal accumulation of mononuclear cells as reported,[8] and a variable number of eosinophils were observed between 7 and 30 days after infection with VTP (Fig. 4d).

DISCUSSION

The present data indicate that in the susceptible C4D guinea-pig, cutaneous infection with VTP induces a very early Th2 differentiation pathway with a concomitantly low or negligible Th1 immune response. Low levels of IL-1, IL-12 and TNF-α mRNA in experimental and control animals cannot be attributed to the lack of sensitivity of the assay, as the three cytokines are readily expressed at high levels by Con A- or lipopolysaccharide- (LPS) stimulated guinea-pig peripheral blood (Fig. 1a and b). On the other hand, expression of a high level of IL-2 in the absence of a defined Th1 response in both experimental animals and in controls, seems to point to a Th0 (innate) T-cell subset as its potential source.[19] Resting T helper precursors normally secrete only IL-2 on first encounter with the antigen and then differentiate into mature effector cells Th1 or Th2.[20] It is feasible that foreign rabbit proteins accompanying the inoculum, rather than the pathogen, are the triggering factor for IL-2. They may also account for the fluctuations in the level of TGF-β expression in control animals. However, none of this seems to affect the overall IL-10 response in the infected animals. It should be stressed that analysis of cytokine mRNA expression by RT-PCR may not correspond to the amount of functional cytokine present, since transcriptional and post-transcriptional mechanisms regulate cytokine activity. Nonetheless, measurement of mRNA levels provides important information on the cytokine expression in the absence of biological reagents to measure functional proteins.

Careful examination of primary syphilitic lesions in rabbit[6] and guinea-pig (Fig. 4) revealed a variable but consistent presence of true eosinophils (larger than pseudo-eosinophils and containing three- to fourfold larger eosinophilic granules), in addition to the mononuclear cell infiltration. In the guinea-pig model, the eosinophilic infiltration could be quite remarkable as many of them appear to be actively degranulating (Fig. 4d). thus providing a potential source of pharmacologically active mediators of inflammation.[21] Since few or no eosinophils were seen in inoculated skin specimens of controls, the possibility of an allergic reaction to rabbits proteins was excluded. While eosinophilia has not been particularly investigated in human syphilitic lesions or blood, an association between Th2 immune response and IgE in the early stages of human syphilis was suggested by two groups of investigators in patients free of any allergic symptoms or signs. Green *et al.*[22] in the USA reported high levels of IgE concentration in serum samples of 50 patients with primary and secondary syphilis, whereas Bos *et al.*[23] in the Netherlands demonstrated significantly higher ($P<0{\cdot}05$) levels of IgE antitreponemal antibodies in patients with primary and secondary syphilis when compared to healthy controls.

There are several experimental models of infection in which susceptible and resistant strains of mice differ in their cytokine expression patterns. For example, infection by *Candida albicans*[24] or *Trypanosoma cruzi*[25] that requires a cell-mediated immune response for resistance results in a higher level of IL-10 in susceptible mice. If one considers that guinea-pigs are less susceptible to VTP than rabbits, and that rabbits in turn, are less susceptible than humans, then it is reasonable to expect that the manifestation and timing of the immunoregulatory response will be subject to changes inherent to the species and host's genetic background.[8] In the guinea-pig model, a predominant Th2 immune response was observed as early as

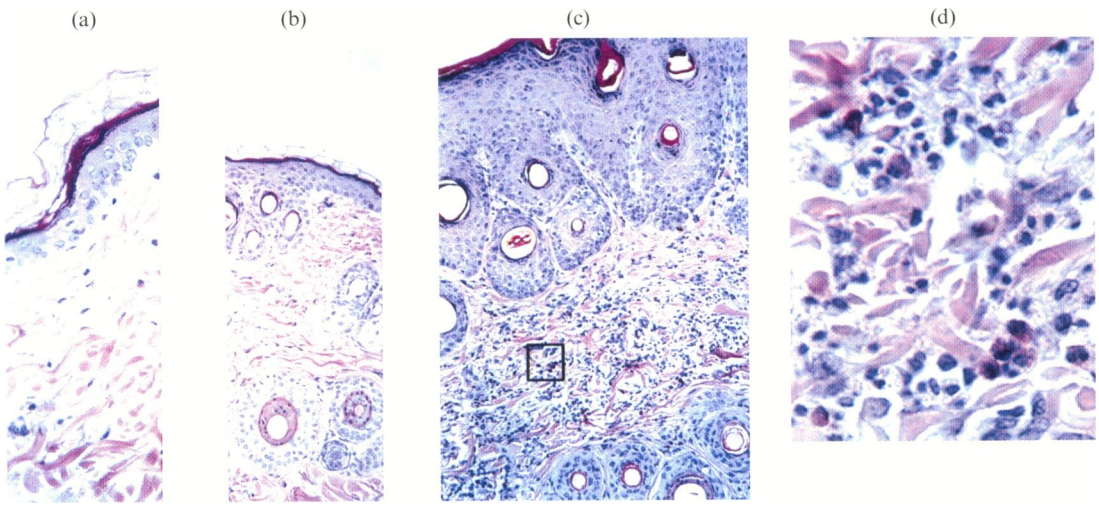

Figure 4. Haematoxylin and eosin staining of guinea-pig skin sections obtained at ≈14 days after injection with ITF (a) (×200), HKTP/ITF (b), or infected with VTP/ITF (c) (both ×100). In addition to the infection-induced hypertrophic epidermis and mononuclear cell infiltration, there was a large number of intact and actively degranulating eosinophils (c). An enlargement of the insert in (c) is shown in (d) (×400).

3 days after infection. In the rabbit model, Fitzgerald[26] reported a switch from a Th1 [IL-2, interferon-γ (IFN-γ)] to a Th2 response after 9–14 days of infection. Van Voorhis *et al.*,[27] using skin biopsies obtained from African patients with primary and secondary syphilis, suggested that the predominantly Th1 response (IL-2, IL-12 and IFN-γ) seen in patients with primary syphilis must be responsible for the effective clearance of the pathogen from the site of infection and dissolution of lesions. However, without exception, all biopsies examined by these investigators contained mRNA encoding IL-10 and 25% contained IL-5 (Th2). This is of relevance as a bulk of evidence has indicated that IL-12 is a potent inducer of IL-10 production[28,29] which acts as a negative feedback mechanism to control and limit ongoing T-cell activation. The net result favours survival of the pathogen, prevention of severe tissue damage, and autoimmune responses.[19,30]

In conclusion, in syphilis infection, the early and profuse humoral response,[31] the delayed acquisition of resistance to reinfection[2] and the protracted and incomplete elimination of the pathogen from the site of inoculation and distant organs[6,32] are all consistent with an early switch to an ineffective Th2 response.

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