Immunohistologic Analysis of the Inflammatory Infiltrates Associated with Osseointegrated Implants

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A panel of monoclonal antibodies was used in an avidinbiotin immunoperoxidase technique to provide an immunohistologic analysis of the inflammatory infiltrates in the gingiva of osseointegrated implants. A total of 27 gingival and 13 interimplant specimens was obtained from 13 patients. Eighteen of the gingival specimens were clinically healthy, while nine showed overt signs of clinical inflammation (GI = 2 or 3). All specimens showed some degree of inflammation histologically, although the size of the inflammatory infiltrate was much greater in the clinically inflamed specimens. There was no significant difference in the proportion of T (50% to 60%) or B (40% to 50%) lymphocytes in either group. The CD4:CD8 ratio also showed no significant difference: 1.6:1 in the healthy group and 2.0 in the inflamed group. Similar ratios were also found in the infiltrates of the interimplant tissue. The immunohistologic analysis suggests that the gingival lesion associated with these implants is a stable, well-controlled response. (INT J ORAL MAXILLOFAC IMPLANTS 1989;4:191-198.)

Key words: immunohistology, osseointegrated implants

The procedure for dental rehabilitation of edentulous patients using fixed prostheses supported by titanium dental implants is now well accepted after extended clinical trials over 15 years.[1] Studies of soft-tissue reactions adjacent to titanium abutments have been made at both clinical[2,3] and histologic levels.[4,5] These investigations have emphasized that the reactions of the "peri-implant" tissues are different from those between the gingival and periodontal tissues.[1-6] It has generally been found that there is little correlation between the inflammatory state of the superficial tissue and any loss of osseointegration.

In recent years, the ability to identify various cell subpopulations infiltrating a chronic inflammatory lesion has resulted in a greater understanding of the pathogenesis of these lesions.[7,8] The original studies of Brandtzaeg[9,10] established that adult periodontitis (AP) tissues contained large numbers of plasma cells. Subsequent studies confirmed the B-cell character of these lesions.[11-13] Contrarily, studies using the experimental gingivitis model and gingivitis in children have shown that the lesion confined to the gingiva is dominated by T cells.[14-17] In both of these lesions, the CD4:CD8 (helper: suppressor) T-cell ratio was approximately 2:1.

At the same time, the majority of T cells displayed HLA-DR and HLA-DQ antigens while the macrophages had an activated phagocytic phenotype. [16,17] These results are very similar to those previously described for the development of a controlled delayed-type hypersensitivity (DTH) reaction in the skin[18] and suggest that, like DTH, the lesion confined to the gingiva is a well-controlled immunologic response. In contrast, the CD4:CD8 ratio in AP lesions is approximately 1:1,[19,20] suggesting a local immunoregulatory imbalance. When taken together, these results support the concept that "stable" periodontal disease is predominantly a T-cell response, while "progressive" disease is mainly a B-cell response[21] and possibly associated with a local immunoregulatory imbalance.[22]

As yet, no immunohistologic study of the reaction adjacent to osseointegrated titanium implants has been reported. The lack of correlation between the degree of inflammation and the loss of integration with these implants may be because of the development of a lesion similar to the "stable" lesion of naturally occurring periodontal disease. Therefore, the aim of the present study was to characterize the cell types infiltrating the gingiva associated with osseointegrated implants so as to allow a comparison with naturally occurring periodontal disease.

Materials and Methods

Tissue. A total of 27 gingival (adjacent to implant) and 13 interimplant specimens was obtained from 14 subjects. Eighteen of the adjacent gingival specimens were clinically healthy (Loe and Silness gingival index of 0 or 1), while nine showed overt signs of clinical inflammation (gingival index of 2 or 3). Inflammation of the interimplant tissue was assessed subjectively by clinical criteria. Brånemark System implants (Nobelpharma AB, Gothenberg, Sweden) were used.

The biopsies were obtained under local anesthesia. Local anesthetic agent (Xylocaine 2% with 1:80,000 adrenalin) was injected into the mucobuccal sulcus at least 1 cm distal to the proposed biopsy site to minimize local anesthetic infiltration into the biopsy. The biopsy was taken between fixtures and included the periosteum where possible. The tissue was oriented in exactly the same way for all patients (Figs la and lb) and immediately immersed in embedding solution (OCT, Naperville, Illinois), quenched in liquid nitrogen, and stored in a freezer at – 70 °C until sectioning. Sutures were placed to achieve primary closure and all biopsy sites healed uneventfully.

Immunohistology. More than 30 serial 6-μm cryostat sections were cut from each specimen, air dried for 2 hours, fixed in equal parts chloroform/acetone for 5 minutes, and stored at –20 °C.[8] A panel of monoclonal antibodies (MoAb) with well-defined specificities was used (Table 1).

After rehydration in phosphate-buffered saline (PBS), pH 7.2, sections were incubated with the primary MoAb at a 1/10 dilution followed by a 1/20 dilution of affinity-purified-biotinylated-goat-anti-mouse immunoglobulin (Vector

Laboratories, Burlingame, California) and finally with a 1/50 dilution of horseradish peroxidase-conjugated streptavidin (Amersham Australia, Pty Ltd, Strawberry Hills, New South Wales). Each incubation was carried out for 30 minutes at room temperature, followed by washing for 10 minutes in PBS. The peroxidase was developed using 0.05% 3.3' diaminobenzidine (Sigma Chemical Co, St Louis, Missouri) in tris-HCL buffer (pH 7.6) containing 0.001% hydrogen peroxide.[23] Nuclei were counterstained with Mayer's hematoxylin. Optimal dilutions of each layer were predetermined using frozen sections of human tonsil. Tonsil sections also served as positive controls. Negative controls included the use of PBS; normal mouse serum; and an irrelevant monoclonal antibody (FN_4/BA_4), which reacts with an antigen on *Fusobucterium nucleatum,*[24] in place of the primary monoclonal antibodies.

Cell Analysis. The number of antigen-positive cells was determined by counting the positive cells within a standard high-power field using an ocular grid (0.01 mm^2). The number of fields varied between specimens depending on the size of the lesion. However, the entire infiltrate in each specimen was examined and the results were presented as the mean number (±standard error [SE]) of antigen-positive cells per 0.01 mm^2. Data were analyzed using the one-way analysis of variance (ANOVA).

Results

Histology. Inflammatory infiltrates were present in all adjacent gingival specimens, even in those classified as clinically healthy. The lesions were located perivascularly subjacent to the crevicular epithelium. The size of the infiltrate appeared larger in the clinically inflamed specimens. The inflammatory infiltrate was composed predominantly of lymphocytes and macrophages, with morphologically identifiable plasma cells constituting less than 10% of the infiltrating population (Figs 2a and 2b).

Immunohistology. *Lymphocytes.* In both the adjacent gingival and interimplant specimens, T and B lymphocytes were identified (Figs 3a and 3b), and in each group of specimens the majority of T cells was CD4-positive (Figs 4a and 4b). The mean numbers (± SE) of T, B, and T-cell subsets in the gingival infiltrates are shown in Table 2. While there was a statistically significant ($P < 0.05$) increase in cell numbers in clinically inflamed specimens compared to the clinically healthy ones, there was no significant difference ($P > 0.05$) in the proportion of each cell type (Table 2). Similarly, there was no significant difference ($P > 0.05$) in the mean CD4:CD8 ratio between the two groups of specimens: 1.64 ± 0.31 in the healthy group and 2.02 ± 0.56 in the inflamed group.

Similar results were found for the interimplant lesions, with a statistically significant increase ($P < 0.05$) in each cell type in the inflamed specimens. However, there was no significant difference between the healthy and inflamed groups in the proportion of each cell type ($P > 0.05$). The mean CD4:CD8 ratio also was not

significantly different between the two groups (Table 3).

Although substantial numbers of B cells were present (Figs 2a and 2b) in both the adjacent gingival and interimplant specimens, T cells predominated and the CD4:CD8 ratio was between 1.5 and 2.0 (Figs 4a and 4b).

Langerhans Cells. Analysis of the intraepithelial CD1-positive Langerhans cells revealed no significant differences between the mean number of Langerhans cells in either inflamed or clinically healthy tissues from either site (Fig 5, Table 4).

HLA Class II Positive Cells. The mean number (±SE) per 0.01 mm^2 of HLA class II positive cells (B cells, macrophages, antigen-presenting cells, and activated T cells) is shown in Table 5. There was a statistically significant difference in the number of class II positive cells between the healthy and inflamed groups at each site ($P < 0.01$). While the percentage of DR-positive cells that were also DP-positive was relatively constant in the adjacent gingival specimens, there was a reduced percentage of DR-positive cells expressing DQ. At the interimplant sites, almost 100% of DR-positive cells in the inflamed groups were coexpressing both DP and DQ.

Discussion

The present study has shown that the inflammation in the gingiva adjacent to osseointegrated implants is a lymphocyte/ macrophage infiltrate with very few plasma cells. The lesion may increase in size such that it becomes clinically evident, but in so doing it remains relatively constant in the proportion of T cells, B cells, and T-cell subsets. It is interesting that inflammation was present in all specimens despite the lack of clinically overt inflammation in the majority.

In proportion, T cells dominated the lesions, although there were substantial numbers of B cells present. The lack of plasma cells, however, suggests that activation of the B-cell population was well controlled. This concept is further supported by the consistent CD4:CD8 ratio between 1.5 and 2.0:1. This ratio is similar to that in peripheral blood and regional lymphatic tissue,[7] and is also similar to that found in DTH[18] and the putative stable periodontal lesions, gingivitis in children,[17] and experimental gingivitis.[16]

There is generally an increase in the number of intraepithelial Langerhans cells in gingival inflammation.[16,17] The lack of any increase in Langerhans cells numbers observed in the present study was therefore surprising. Nevertheless, activity within the epithelium was evident by the consistent finding of marked epithelial hyperplasia in the interimplant regions. The reasons for this hyperplasia and the underlying inflammatory reaction are not clear, and further work is necessary to determine the mechanisms of this response.

HLA class II antigens are expressed on B cells, activated macrophages, antigen-presenting cells, and activated T cells. In the present study, double labeling

experiments were not performed. Thus it was not possible to identify individual class II positive cells. However, analysis of the cell numbers would suggest that only about 10% of the T cells are expressing HLA-DR antigens. HLA class II antigens are induced by IFN-γ[25-27] and are a late activation antigen on T cells. Hence, if only 10% of T cells are expressing HLA class II antigens, it would suggest that either they are in an early activation stag or, more likely, are not being activated locally. This latter contention would seem to be supported by the other immunohistologic evidence, all of which suggest that these lesions associated with osseointegrated implants are relatively stable, well-controlled responses. Further studies using double labeling are required to identify these class II antigen-bearing cells precisely.

Conclusion

Overall, the findings described in the present study are very similar to those previously described for the development of a controlled DTH reaction in the skin,[18] gingivitis in children,[17] and the development of experimental gingivitis.[16] This observation suggests that gingivitis associated with osseointegrated implants, like the aforementioned lesions, is a well-controlled immunologic response and represents a stable periodontal condition. Further work is now necessary to determine the immunohistologic profile of the "periodontal (peri-implant) lesion" associated with failure of osseointegrated implants.

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Table 1 Monoclonal Antibodies

Antibody		Specificity	Source
Leu 4	CD3	Pan T lymphocyte	Beeton-Dickinson*
Leu 14	CD22	Pan B lymphocyte	Becton-Dickinson*
Leu 2	CD8	Suppressor/Cytotoxie T lymphocytes	Becton-Dickinson*
Leu 3	CD4	Helper/inducer T lymphocytes	Becton-Dickinson*
Anti-DR		HLA-DR nonpolymorphic antigens	Becton-Dickinson*
Anti-DP		HLA-DP nonpolymorphic antigens	Becton-Dickinson*
Leu 10		HLA-DQ nonpolymorphic antigens	Becton-Dickinson*
OKT6	CD1	Cortical thymocytes Langerhans cells	Orthomune†

**Becton-Dickmson Monoclonal Center Inc, Mountain View, California.*
†Orthomune, Ortho Diagnostics, Australia.

Table 2 Analysis of Lymphocytic Infiltrates in the Gingiva Adjacent to the implants

	T cells CD3		B cells CD22		Helper T cells CD4		Suppressor T cells CD8		Mean ratio
Tissue	No.	%	No.	%	No.	%	No.	%	CD4:CD8
Healthy n = 18	14.22 (± 2.90)	59.67	9.61 (± 2.01)	40.33	8.33 (± 1.19)	37.05	6.83 (± 1.09)	28.66	1.64 ± 0.31
Inflamed n = 9	30.33 (± 7.51)	54.38	25.44 (± 7.03)	45.62	21.33 (± 6.32)	38.25	12.56 (± 2.5)	22.52	2.02 ± 0.56
Total n = 27	19.59 (± 3.49)	56.85	14.87 (± 3.06)	43.15	13.00 (± 2.52)	37.72	8.74 (± 1.22)	25.36	1.77 ± 0.28

Mean number (± SE) of T, B, and T-cell subsets per 0.01 mm^2.
n = number of specimens.

Table 3 Analysis of Lymphocytic Infiltrates in the Interimplant Tissue Midway Between Implants

	T cells CD3		B cells CD22		Helper T cells CD4		Suppressor T cells CD8		Mean ratio
Tissue	No.	%	No.	%	No.	%	No.	%	CD4:CD8
Healthy	11.89	64.06	6.67	35.94	7.11	38.31	5.33	28.72	1.48
n = 18	(± 4.09)		(± 1.53)		(± 1.60)		(± 0.75)		(± 0.32)
Inflamed	40.75	69.07	18.25	30.93	20.33	34.46	9.33	15.81	2.01
n = 9	(± 22.05)		(± 7.19)		(± 7.62)		(± 2.84)		(± 0.62)
Total	20.77	67.00	10.23	33.00	12.54	40.45	6.08	19.61	1.61
n = 27	(± 8.23)		(± 2.87)		(± 3.29)		(± 0.99)		(± 0.29)

Mean number (± SE) of T, B, and T-cell subsets per 0.01 mm^2.
n = *number of specimens.*

Table 4 Analysis of Langerhans Cells Adjacent to and Midway Between Implants

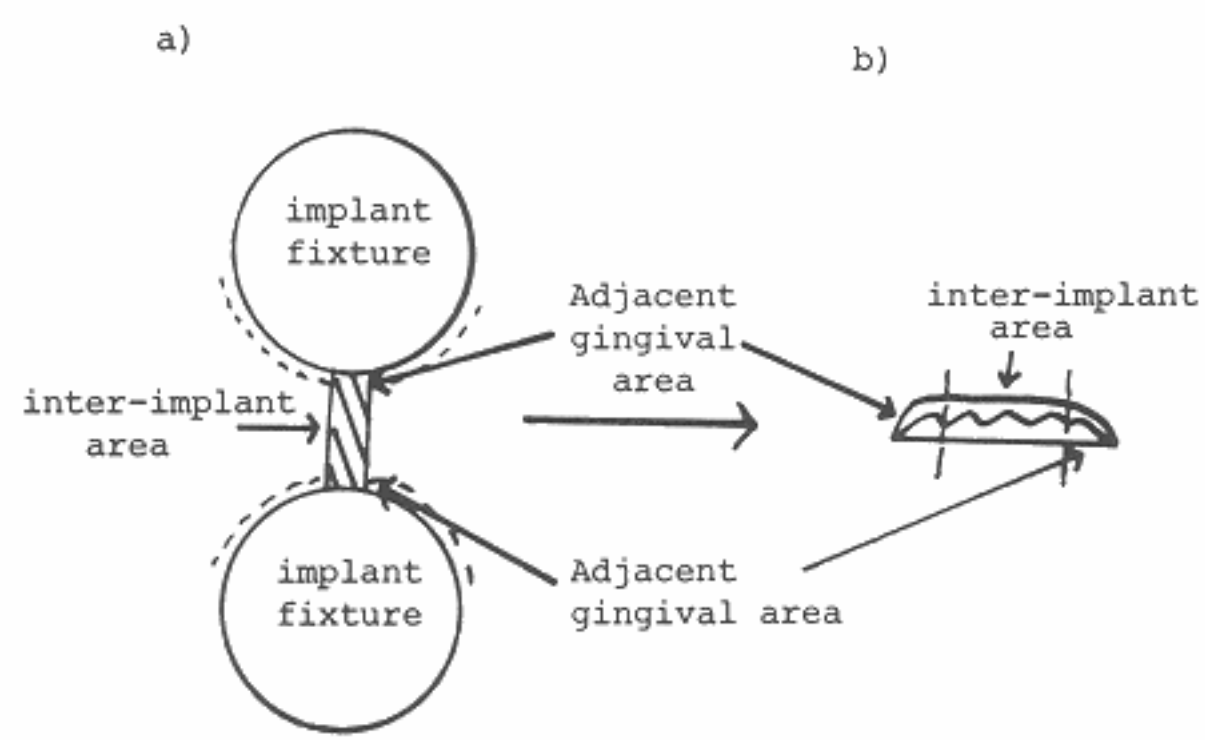
	Gingival		Interimplant	
Tissue	n	CD1	n	CD1
Healthy	18	6.06 (± 0.57)	9	6.22 (± 0.58)
Inflamed	9	5.89 (± 0.97)	4	4.00 (± 0.50)
Total	27	6.00 (± 0.50)	13	5.54 (± 0.52)

Mean number (± SE per 0.01 mm^2).
n = *number of specimens.*

Table 5 Analysis of Cells Expressing HLA Class II Antigens

	Gingival				Interimplant			
Tissue	n	DR	DQ (% of DR)	DP (% of DR)	n	DR	DQ (% of DR)	DP (% of DR)
Healthy	18	12.50 ± 1.42	10.06 ± 1.21 (80.5)	8.39 ± 1.21 (67.1)	9	9.11 ± 2.05	6.33 ± 1.05 (69.5)	5.56 ± 1.01 (61.0)
Inflamed	9	25.14 ± 4.95	17.33 ± 2.61 (68.9)	16.11 ± 2.02 (64.1)	4	15.33 ± 4.38	15.33 ± 5.08 (100.0)	15.00 ± 4.08 (97.8)
Total	27	16.84 ± 3.37	12.48 ± 1.56 (74.1)	10.96 ± 1.26 (65.1)	13	10.67 ± 2.04	9.83 ± 2.30 (92.1)	7.91 ± 1.73 (73.0)

Mean ± SE per 0.01 mm^2.
n = *number of specimens.*



Figs. 1a and 1b (a) Diagrammatic representation of the biopsy site. Dotted line illustrates the adjacent gingival areas; cross-hatched, the interimplant area. (b) Longitudinal orientation of biopsy, enabling examination of the two adjacent gingival areas and the interimplant area.

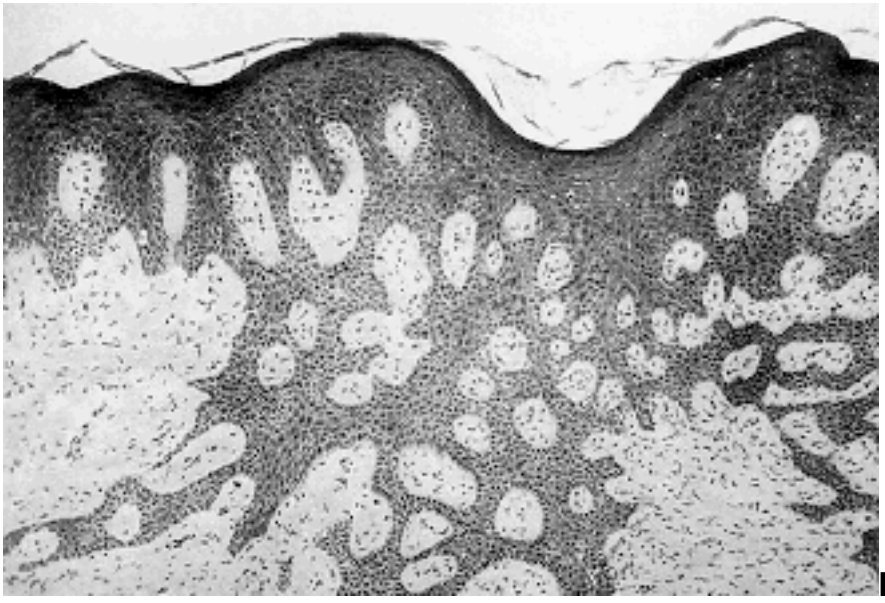


Fig. 2a Interimplant area showing hyperplastic epithelium (H&E, original magnification × 80).

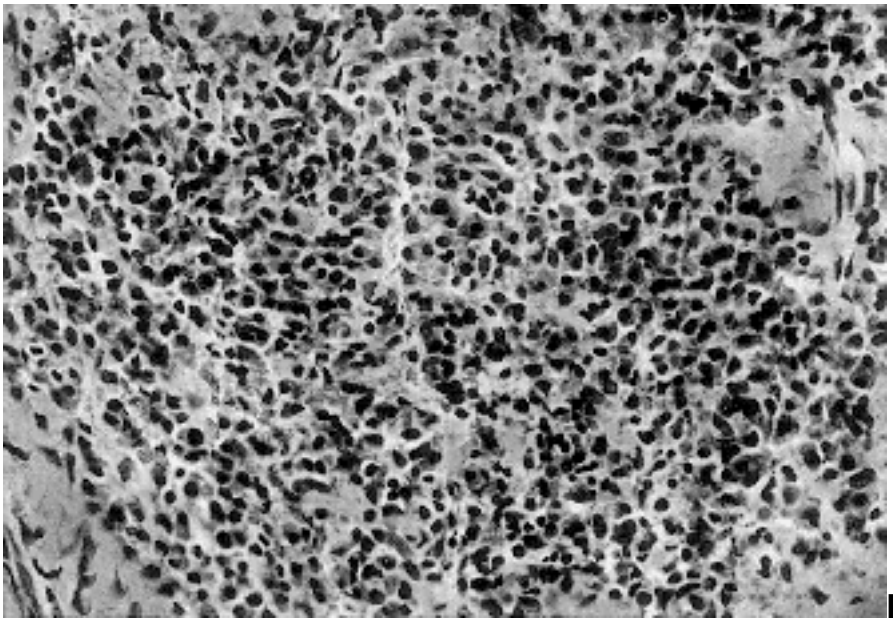


Fig. 2b Inflammatory lesion in gingiva adjacent to implant showing a dense lymphocyte/macrophage infiltrate (H&E, original magnification × 200).

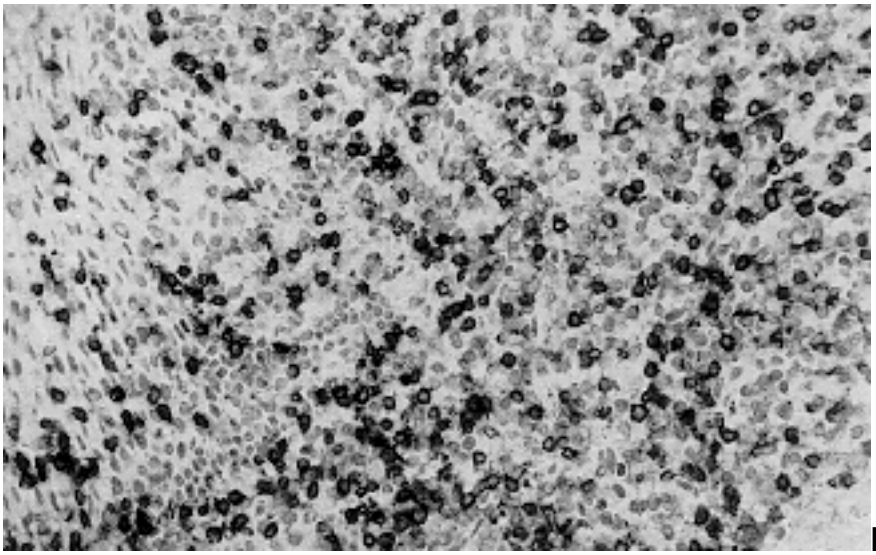


Fig. 3a CD3-positive T cells in an infiltrate adjacent to an implant (original magnification × 200).

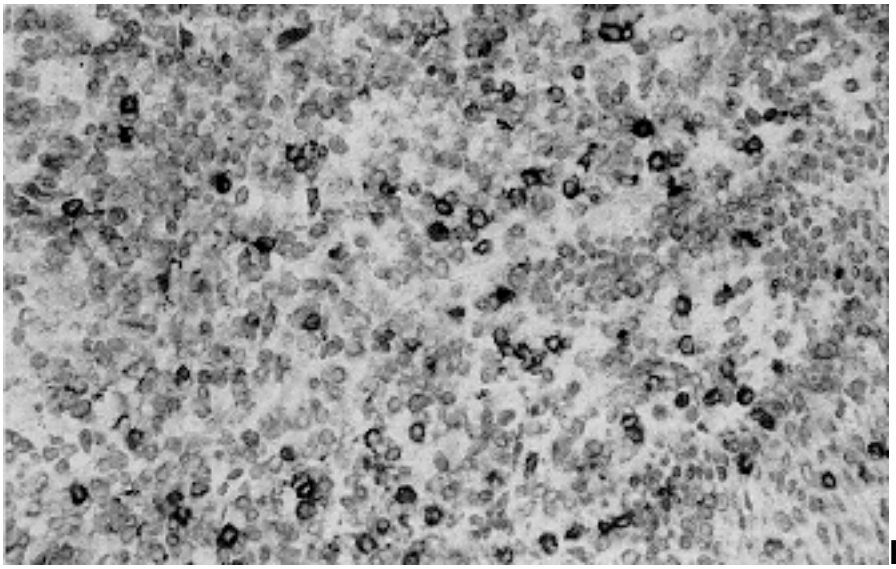


Fig. 3b Leu-14-positive B cells in an infiltrate adjacent to an implant (original magnification × 200).

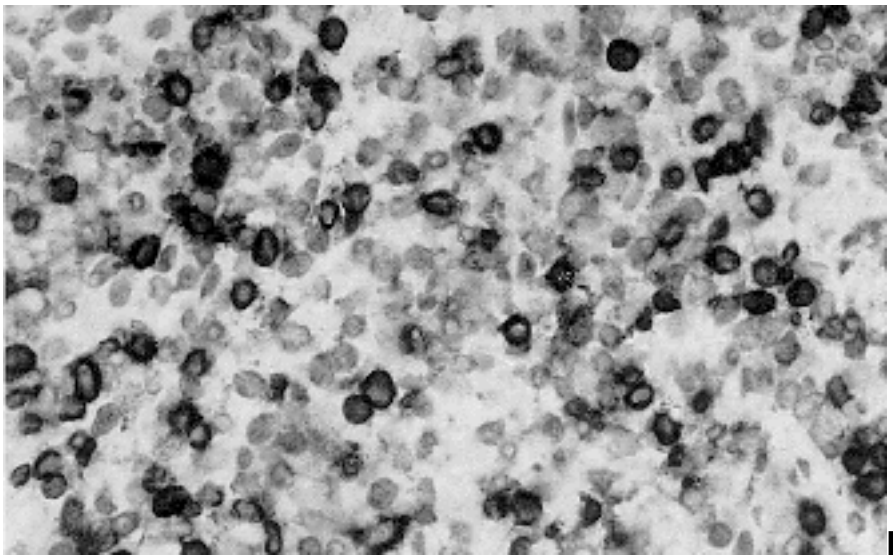


Fig. 4a CD4-positive (helper/inducer) T cells in an infiltrate adjacent to an implant (original magnification × 320).

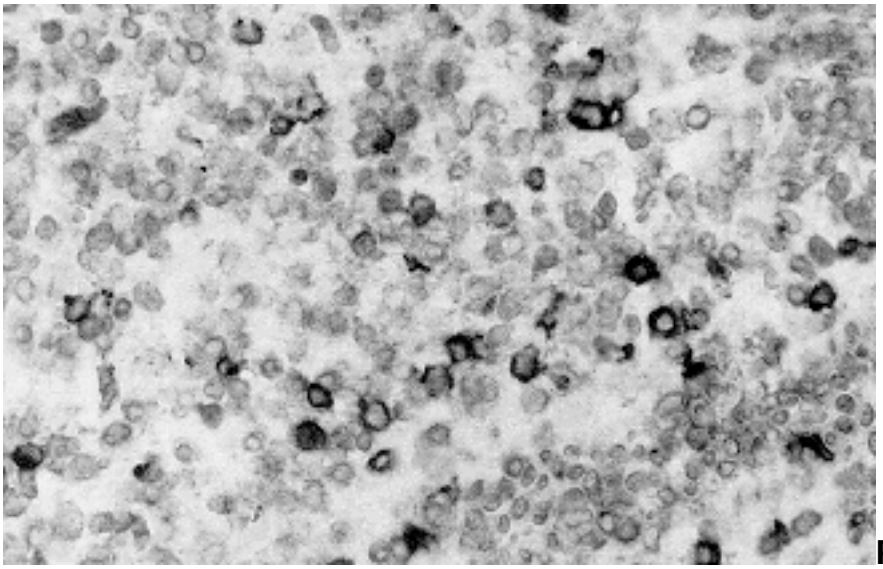


Fig. 4b CD8-positive (suppressor/cytotoxic) T cells in an infiltrate adjacent to an implant (original magnification × 320).

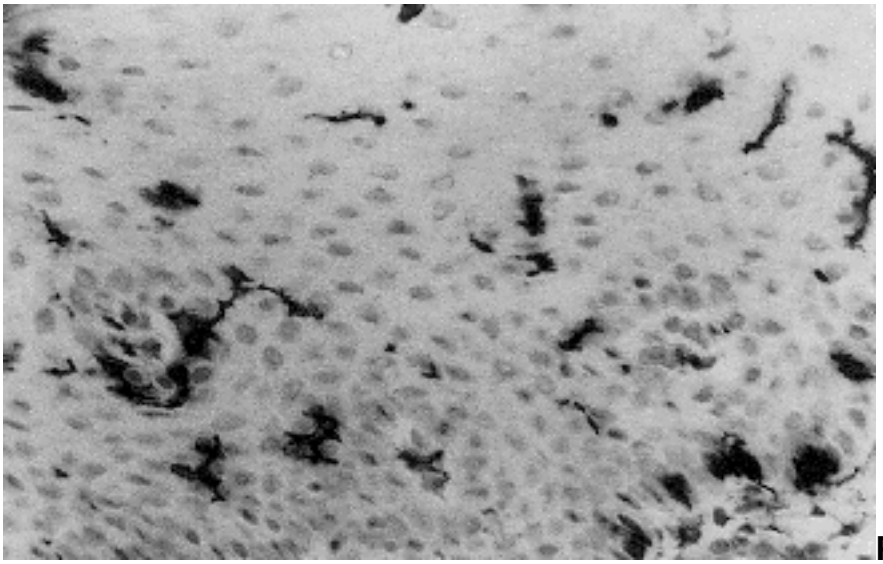


Fig. 5 High-power view of dendritic CD1-positive Langer-hands cell (original mangification × 320).