In situ characterization of mononuclear cells in marginal periodontitis of patients with Down's syndrome

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An indirect immunofluorescence technique on cryostat sections was used to study the cellular composition in chronic marginal periodontitis (CMP) of patients with Down's syndrome (DS). The findings were compared with CMP lesions in otherwise normal patients (NP). The distribution and amount of $CD22^+$ cells (B lymphocytes), $CD3^+$ cells (pan T lymphocytes), $CD4^+$ cells (helper T subset), $CD8^+$ cells (suppressor/cytotoxic T subset), and $CD11c^+$ cells (in tissue, mainly monocytes and macrophages) were investigated. Morphologic studies showed a denser inflammatory infiltrate in DS than in NP. Countings showed significant differences in cell distribution ($p = 0.0003$) and cell profiles ($p = 0.0273$) between the two groups. The median $CD4^+/CD8^+$ ratio in DS (2.73) was significantly higher ($p = 0.0024$) than found in gingival inflammatory lesions from NP (1.08). The present study shows that DS patients have a different, more pronounced, immune response in CMP than NP. □ *Inflammation; periodontal diseases; trisomy 21*

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Patients with Down's syndrome (DS) have an increased prevalence of chronic marginal periodontitis (CMP) compared with otherwise normal age-matched control groups or compared with other mentally retarded patients of similar age distribution (1–6). Miller & Ship (7) reported a more rapid progression of CMP on the basis of Russell's Periodontal Index (8).

There is no significant difference in the total number of polymorphonuclear leukocytes in peripheral blood between DS and normal individuals (9). There is, however, an increased turnover of such cells (10) and an increased proportion of immature forms (11). This is thought to result in a reduction of the bactericidal function of the polymorphonuclear leukocytes. An impaired chemotaxis of the polymorphonuclear leukocytes has also been reported (12, 13), and their phagocytic ability has been found to be reduced (9, 14).

The number of B lymphocytes in peripheral blood is normal (15) or reduced (16, 17).

Surveys of the number and distribution of circulating T lymphocytes in persons with DS have given various results. The number of T lymphocytes has been reported to be lower in DS (16). Whittingham et al. (18) suggested that the T-lymphocyte system of institutionalized DS patients is under continuous stress, giving an increase in the number of immature cells. Seger et al. (9) found the transformation capacity of peripheral blood lymphocytes in DS to be reduced with age, probably because of premature aging of the thymus-dependent immune system.

No studies we know of have analyzed inflammatory infiltrates in lesions of CMP in DS. On the basis of studies in CMP from otherwise normal patients (NP) suggestions have been made that a switch from T-lymphocyte to B-lymphocyte domination in the

lesions is a major mechanism of progressive CMP (19, 20). The T lymphocytes and local variations in the cell composition, however, have also been suggested to play important roles in the pathogenesis in CMP (21–25).

The present investigation was undertaken to 1) study the composition of the immunocompetent cells in gingival inflammatory infiltrates in patients with DS, and 2) compare the distribution of such cells in DS patients with gingival infiltrates from periodontitis in otherwise normal individuals.

Materials and methods

Tissues

In connection with other necessary dental treatment (periodontal disease, caries, extractions, and so forth) marginal gingival biopsy specimens were collected from gingivectomies in 16 DS patients with clinically manifest periodontal disease, aged 20 to 38 years (mean age ± SD, 29.8 ± 4.7 years). There were 10 female and 6 male patients. Twelve similarly treated specimens from otherwise normal patients with CMP, aged 31 to 72 years (mean age ± SD, 40.9 ± 11.6 years), were used as controls. All specimens were taken where there was bleeding on probing and pocket depth of 4 mm or deeper.

Biopsy specimens were cut in a plane parallel to the long axis of the teeth and were orientated so that the pocket epithelium (PE), connective tissue (CT), and oral gingival epithelium (OGE) were present in the same section. The specimens were mounted on copper blocks with Tissue Tek®, snap-frozen in isopentane precooled to −140°C in liquid nitrogen, and stored at −80°C.

Cryostat sections were cut at 4 µm and mounted on gelatin-coated glass slides. The sections were briefly dried in air, fixed in acetone at room temperature for 10 min, and rinsed in phosphate-buffered saline, pH 7.2 (PBS), for 15 min before incubation.

Monoclonal antibodies and antisera

A panel of monoclonal antibodies with defined specificities were used to identify CD22 (B lymphocytes), CD3 (T lymphocytes), CD4 (mainly helper T lymphocytes), CD8 (mainly suppressor/cytotoxic T lymphocytes) and CD11c (mainly monocytes/macrophages) (Table 1).

The monoclonal antibodies used were diluted in accordance with the results of dilution tests done in our laboratory (Table 1). Cryostat sections were incubated with monoclonal antibody overnight at room temperature in a moist chamber (26, 27). After having been washed in PBS, all sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (RAMIg) (Dakopatts, Copenhagen, Denmark; code F232) diluted 1:20 (10 µg/ml antibody) in PBS, containing 25% pooled human serum (PHS) for 30 min. The sections were further washed and incubated with FITC-conjugated goat anti-rabbit (GARIg) (Behring Diagnostica, Marburg, Germany; code OTKF 05), diluted 1:20 (5 µg/ml specific antibody) in PBS containing 25% PHS for 30 min. After a final wash-

Table 1. Monoclonal antibodies used

Monoclonal antibodies	Predominant reactivity	Antigen cluster designation	Protein concentration, µg/ml
Leu 14	B lymphocytes	CD22	0.05
Leu 4	Pan T lymphocytes	CD3	0.01
Leu 3a + Leu 3b	Helper T subset	CD4	0.01
Leu 2a	Suppressor/cytotoxic T subset	CD8	0.03
Leu M5	Monocytes/macrophages	CD11c	0.1

All from Becton Dickinson, Sunnyvale, Calif., USA.

ing, sections were mounted in 1,4-diaminobenzene/glycerol and examined under a Leitz Orthoplan microscope equipped with a 150-W xenon lamp and filter block K2.

Parallel sections were stained with hematoxylin, eosin, and safranin (HES) for orientation and more general evaluation of morphology.

Control sections were incubated with PBS or normal mouse serum instead of the monoclonal antibodies.

Numerical cell density

Counting of the inflammatory cells was done in fields 470 μm in diameter (0.17 mm^2) within each of four fields in the inflamed CT subjacent to the PE (Fig. 1). All countings were done by one investigator (P. D. C. Søhoel), who had been calibrated with two of the coinvestigators (A. C. Johannessen and R. Nilsen). The number of cells per square millimeter was calculated.

Consecutive sections with appropriate morphology were obtained in 11 of the 16 specimens. In consecutive sections from these specimens $CD22^+$ cells, $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, or $CD11c^+$ cells were counted, and the $CD4^+/CD8^+$ ratio was calculated.

Statistical analysis

To establish whether there were statistically significant differences between DS and NP, between the different cell types, and between fields, we initially performed analysis of variance and covariance with repeated measures. The data, not following a normal distribution, were logarithmically transformed. We used DS and NP as grouping factors, with cell types and fields as dependent variables.

On the basis of the results of these initial analyses, we used Mann-Whitney U-tests to investigate further the relations between means of cell countings for the four fields.

Finally, the cell profile was analyzed with analysis of variance on pairs of logarithmically transformed means of cell countings.

All statistical methods were two-tailed.

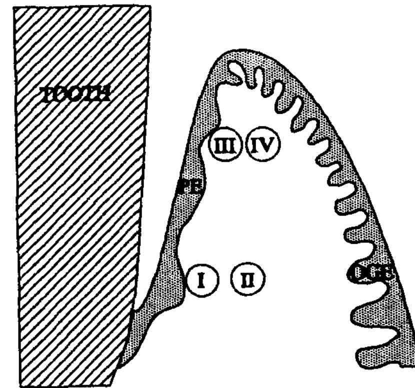

Fig. 1. Fields in which cell counting was performed; field diameter, 470 μm. PE = pocket epithelium; OGE = oral gingival epithelium.

The statistical software package used was BMDP® for PC—Release 88.2 (by BMDP Statistical Software Inc., Los Angeles, Calif., USA), programs 2V and 3D.

Results

Morphology

Examination of the HES-stained sections from DS patients showed a CT infiltrated mainly by small, round, lymphocytes and some plasma cells, but very few polymorphonuclear leukocytes. The inflammatory infiltrates were extremely dense, often concealing the morphology of the CT and involving the entire CT. In sections in which there were differences in the density of infiltrating cells, the CT subjacent to the PE was the most densely infiltrated part of the sections. In some sections both the OGE and the PE were thickened.

Immunohistochemical studies

A very dense infiltrate of $CD22^+$ cells was found in DS specimens (Fig. 2). These cells were distributed differently from section to section. In some sections focal aggregates of cells occupied the CT, whereas other sections demonstrated a more even distribution of positive cells throughout the CT. In two

of seven specimens from NP, $CD22^+$ cells were very scarce, whereas four of the specimens had one or two large aggregates of $CD22^+$ cells.

In the DS material aggregates of $CD3^+$ cells were seen throughout the infiltrated CT. The largest aggregates of positive cells were seen subjacent to the PE (Fig. 2), whereas smaller aggregates were encountered centrally in the CT. Underneath the OGE the cellular infiltrates were less dense than near the PE. $CD3^+$ cells were also encountered within the PE. In the NP sections a narrow band and scattered aggregates of $CD3^+$ cells were seen subjacent to the OGE. Deeper in the CT scattered positive cells were seen, the infiltrate becoming denser subjacent to the PE, where aggregates of positive cells also were encountered. Some positive cells were seen in the epithelia of NP.

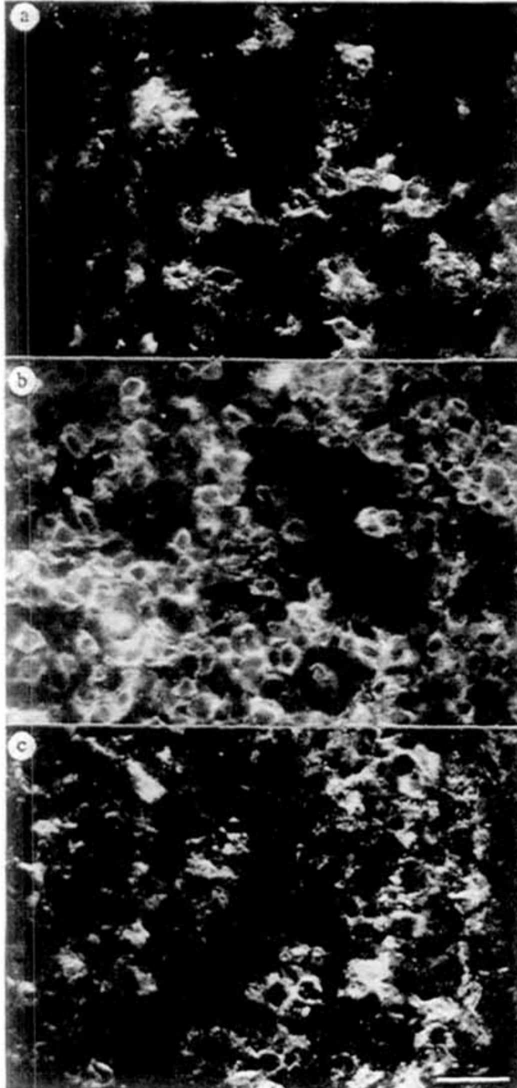

Fig. 2. Immunofluorescence staining of gingival specimens, showing dense infiltrates of mononuclear inflammatory cells in gingiva from a Down's syndrome patient. Areas shown are all from the connective tissue subjacent to the pocket epithelium. 2a. $CD22^+$ cells (B lymphocytes). 2b. $CD3^+$ cells (T lymphocytes). 2c. $CD11c^+$ cells (mainly monocytes/macrophages). White bar = 10 μm.

In DS $CD4^+$ cells filled the entire width of the CT. A denser infiltrate of $CD4^+$ cells was regularly seen subjacent to the PE (Fig. 3). In the CT subjacent to the OGE scattered cells were seen, the infiltrate often increasing in number towards the marginal part of the OGE. In NP a band of positive cells was seen subjacent to the OGE, often becoming denser towards the marginal part of the gingiva. Subjacent to the PE the infiltrating $CD4^+$ cells also were more numerous in the marginal part of the gingiva, where aggregates of positive cells could be seen. Scattered positive cells were seen in the more central parts of the CT. Some positive cells were seen in the epithelia.

Compared with $CD4^+$ cells, a more modest, but still continuous distribution of $CD8^+$ cells was evident in the central parts of the CT of DS. Aggregates of positive cells were seen subjacent to the OGE and subjacent to the PE (Fig. 3). $CD8^+$ cells were also encountered within the basal cell layers of the OGE. In NP a band of positive cells was seen subjacent to the OGE, positive cells becoming more scarce in the central parts of the CT. Subjacent to the PE larger numbers of positive cells were encountered, being more abundant towards the bottom of the periodontal pocket. Positive cells were also encountered within the epithelia.

$CD11c^+$ cells were found as a continuous infiltrate throughout the CT of DS sections, The number of positive cells was, however, generally greater subjacent to the epithelia, both the PE (Fig. 3) and the OGE. Positive

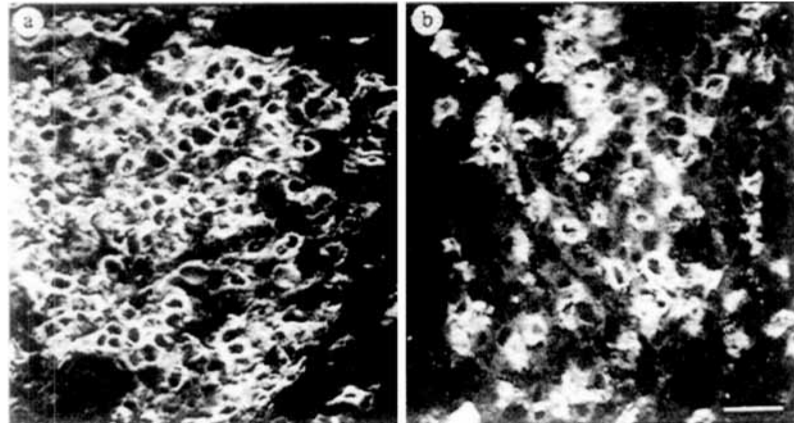

Fig. 3. Immunofluorescence staining of gingival specimens showing dense infiltrates of a) $CD4^+$ (T helper lymphocytes) and b) $CD8^+$ cells (T suppressor/cytotoxic lymphocytes) in gingiva from a Down's syndrome patient. Areas shown are all from the connective tissue subjacent to the pocket epithelium. Note that the density of $CD4^+$ cells is greater than that of $CD8^+$ cells. White bar = 10 μm.

cells were also encountered within the epithelia. In NP positive cells were seen in contact with the basal cell layers of the OGE and PE, and aggregates of positive cells were seen subjacent to the PE, becoming more scarce towards the central parts of the CT.

Sections incubated with FITC-conjugated RAMIg and FITC-conjugated GARIg alone were negative, as were sections incubated with normal mouse serum as the first layer.

Numerical cell density

Results from the cell countings are presented in Tables 2 and 3.

Statistical analysis

The analysis of variance and covariance with repeated measures on the logarithmically transformed data from the cell countings showed a statistically significant difference between DS and NP ($p = 0.0003$) and between the profiles of cell countings for the two groups ($p = 0.0273$). There was, however, not a statistically significant difference between fields ($p = 0.1528$).

The U-tests showed statistically significant differences between DS and NP for $CD3^+$ cells ($p = 0.0035$), $CD11c^+$ cells ($p = 0.0001$), $CD4^+$ cells ($p = 0.0002$), and $CD8^+$ cells ($p = 0.0167$), whereas $CD22^+$ cell countings for the two groups were not statistically different ($p = 0.1165$). There was also a statistically significant difference between $CD4^+/CD8^+$ ratios of the two groups ($p = 0.0024$).

The analysis of variance between pairs of cells showed a statistically significant difference in profile between cell countings from DS and NP for $CD22^+$ cells and $CD11c^+$ cells ($p = 0.0244$) and between $CD3^+$ cells and $CD11c^+$ cells ($p = 0.0102$). There was also a difference between $CD8^+$ cells and $CD4^+$ cells, with a patient group effect ($p < 0.0001$), a total cell effect ($p = 0.0001$) and cell effect by group ($p = 0.0006$).

Discussion

This study was based on the use of tissue otherwise discarded. We therefore felt we could not burden the patient with the use of more thorough clinical variables than are common in general practice, especially since there are at present no clinically applicable methods available to determine the onset, peak, and termination of active destruction with any degree of confidence (28, 29).

The much more dense infiltrate of inflammatory cells seen in the HES-stained sections of CMP from DS patients than in NP shows that there is a different inflammatory pattern in the two groups. The over-

Table 2. Distribution (cells/mm^2) of CD22$^+$ cells (Down's syndrome (DS, $n = 11$) and normal patients with periodontitis (NP, $n = 7$)), CD3$^+$ cells (DS, $n = 11$; NP, $n = 11$), and CD11c$^+$ cells (DS, $n = 11$; NP, $n = 10$) in fields I through IV, represented by median, lower quartile (Q1), and upper quartile (Q2)

	Field I		Field II		Field III		Field IV		All fields	
	DS	NP	DS	NP	DS	NP	DS	NP	DS	NP
CD22$^+$										
Median	653	39	550	9	500	124	400	9	533	285
Q1	240	0	370	0	376	21	120	0	430	41
Q2	990	291	1190	488	740	244	1290	865	1015	601
CD3$^+$										
Median	2270	388	1420	206	1470	582	1450	576	1453	577*
Q1	1020	235	680	41	700	324	750	188	1280	381
Q2	2800	759	1870	935	2130	800	1859	1759	2420	718
CD11c$^+$										
Median	1090	171	1560	106	1212	242	1140	121	1278	175†
Q1	610	53	990	41	730	182	841	112	895	119
Q2	1750	382	1647	200	1660	406	1470	206	1554	258

* Statistically significant difference between DS and NP ($p = 0.0035$, two-tailed).
† Statistically significant difference between DS and NP ($p = 0.0001$, two-tailed), Mann–Whitney U-test.

all finding that CD22$^+$ cells, CD3$^+$cells, and CD11c$^+$ cells appeared in much larger numbers in CMP of DS than in NP (Fig. 4) confirms this suggestion.

There were statistically significant differences between DS and NP for all cell types studied except for CD22$^+$ cells. As can be observed in Table 2, there were twice as many CD22$^+$ cells in DS as in NP. Two of eight sections from the NP group, however, did not show any CD22$^+$ cells in the fields where countings were done. In the logarithmic transformation these were then recorded as missing, therefore giving a higher

Table 3. Distribution (cells/mm^2) of CD4$^+$ cells (Down's syndrome (DS, $n = 11$) and normal patients with periodontitis (NP, $n = 10$)), CD8$^+$ cells (DS, $n = 11$; NP, $n = 9$), and CD4$^+$/CD8$^+$ cell ratio (DS, $n = 11$; NP, $n = 8$) in fields I through IV, represented by median, lower quartile (Q1), and upper quartile (Q2)

	Field I		Field II		Field III		Field IV		All fields	
	DS	NP	DS	NP	DS	NP	DS	NP	DS	NP
CD4$^+$										
Median	1424	159	482	62	1359	283	1212	165	1165	265*
Q1	782	88	312	12	1253	41	741	29	985	187
Q2	2118	471	1165	212	2418	612	1559	347	1566	353
CD8$^+$										
Median	629	253	547	59	435	188	500	94	496	212†
Q1	447	129	276	0	247	53	276	29	384	144
Q2	853	341	853	147	576	429	582	171	769	232
CD4$^+$/CD8$^+$										
Median	2.60	0.84	1.69	1.33	3.57	0.87	2.47	0.69	2.73	1.08‡
Q1	1.68	0.64	0.69	0.99	2.27	0.52	1.02	0.21	1.85	0.88
Q2	3.08	1.14	2.28	2.49	5.65	1.10	4.27	1.22	3.60	1.29

* Statistically significant difference between DS and NP ($p = 0.0002$, two-tailed).
† Statistically significant difference between DS and NP ($p = 0.0167$, two-tailed).
‡ Statistically significant difference between DS and NP ($p = 0.0024$, two-tailed), Mann–Whitney U-test.

CELL DISTRIBUTION

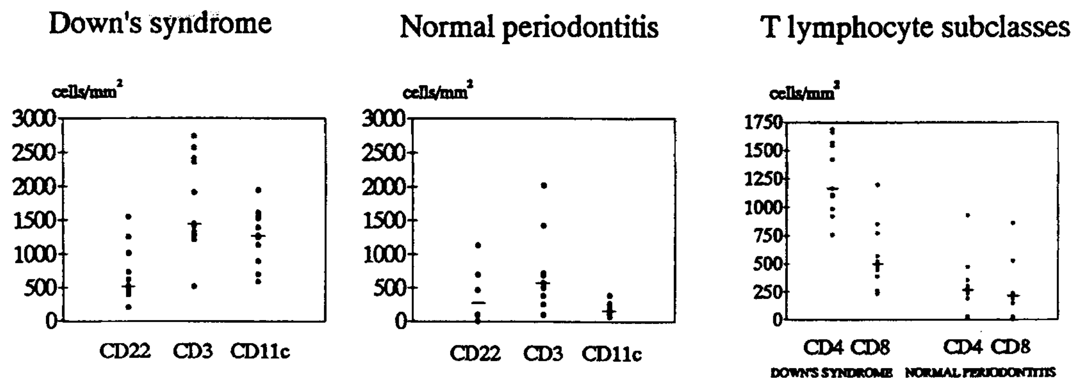

Fig. 4. Scatterplots of mean number of cells in fields I through IV for each patient. $CD22^+$ cells (B lymphocytes), $CD3^+$ cells (T lymphocytes), $CD11c^+$ cells (monocytes/macrophages), $CD4^+$ (T helper lymphocytes), or $CD8^+$ (T suppressor/cytotoxic lymphocytes). Horizontal bar represents the median.

mean $CD22^+$ cell count for NP. In a nonparametric test like the U-test, the magnitude of difference in values between groups disappears.

Compared with CMP in NP the numerically greatest difference in cells per square millimeter was seen for $CD11c^+$ cells, followed by the $CD4^+$ cells (Fig. 4.). However, the number of the other cell types was also substantially higher in DS. These results differ from studies analyzing leukocytes of peripheral blood (15–17), suggesting an active selection of cells in the lesions.

The very high number of $CD22^+$ cells and the high $CD4^+/CD8^+$ ratio (Table 3) in DS could reflect important indicators of an active tissue destruction. Such a correlation has been suggested to occur in lesions of active periodontitis in NP (20, 22, 30–32). However, the mechanism behind this theory is uncertain, and the monitored findings are also thought to reflect a proliferation as a consequence of a nonspecific stimulation (33, 34). Altered immune regulation has previously been considered to be a factor in the pathogenesis of human periodontitis. Juvenile periodontitis has been shown to have an elevated $CD4^+/CD8^+$ ratio in tissue lesions (35). A shift in the ratio to higher values has also been reported in several other active inflammatory lesions compared with nonresponsive sites (36–38). The $CD4^+/CD8^+$ ratio found in our NP group is in accordance with results presented by Johannessen et al. (22). From the present findings in DS, demonstrating that the $CD4^+/CD8^+$ ratio in DS was twice as high as reported in gingival lesions from NP, we conclude that there obviously is an abnormal composition of these subclasses, which could reflect alterations in immunoregulation. Our study therefore supports the view of a correlation between local tissue destruction and a high $CD4^+/CD8^+$ ratio.

Although the role of T helper lymphocytes probably is central, a reduced relative number of T suppressor lymphocytes may give rise to the increased B-cell proliferation and thus reflect an improper immune regulation within the CT, as proposed by Okada al. (25, 39).

The greater number of $CD11c^+$ cells seen in CMP of DS than in NP could also explain some of the extensive periodontal destruction seen in DS. This finding is in accordance with findings by Jully et al. (40), who found more macrophages in periodontitis than in gingivitis of normal individuals.

Altered immune reactions of DS, with a high number of immunocompetent cells in general and the high $CD4^+/CD8^+$ ratio, could on this background explain the more

serious and rapidly advancing destructive process seen in DS than in NP. The same mechanisms as proposed to give disease in DS may also account for focal destruction in NP. We do not know, however, whether the DS lesions we have studied represent moments or periods of periodontal destruction or, indeed, whether periodontal disease in DS follows such a pattern of destructive and stable periods as suggested for NP lesions.

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