

Particularity of Local Immunity in the Nasopharynx

Parallel Study of Surface Receptors and Cell-Mediated Immune Responses in Cells Derived from Palatine or Pharyngeal Tonsils and Blood

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Immunological functions of the pharyngeal tonsil, palatine tonsils and blood leucocytes of children undergoing tonsillectomy were evaluated by determining T or B lymphocytes, the response to mitogens, and the cell-mediated immunological responses to tuberculin. In all the test systems used similar results were obtained with cells derived from either the palatine or pharyngeal tonsils. The mean percentage of T lymphocytes was significantly higher in the peripheral blood than in tonsils, but the reverse was true of B lymphocytes. The reaction to PHA was lower in tonsillar cell culture than in blood cell culture, but tonsillar cells reacted better to Con A than blood cells. In lymphocyte transformation tests tonsillar cells reacted to specific antigen (tuberculin) and this reaction was significantly higher than that of the parallelly tested blood lymphocytes. Further, in about 50% of the children tested, tuberculin caused migration inhibition of the mixture containing tonsillar cells and guinea pig peritoneal cells. Surprisingly, nearly identical results were obtained if migration inhibition test was performed with tonsillar cells alone. Consequently, poorly migrating tonsillar cells are nevertheless usable for direct migration inhibition testing.

Key words: cell migration inhibition; immunity, cellular; leukocytes; lymphocyte transformation; mitogens; rosette formation; tonsil; tuberculin test.

Accepted for publication 21 July 1978

Membrane markers and cell-mediated immune reactions are usually studied in human circulating blood lymphocytes. These lymphocytes represent systemic immunity of the whole body. On the other hand, the existence of local immunity, confined to a particular region of the body, is now well documented (35).

The tonsils are accumulations of lymphoid tissue which represents the anatomical basis for local immune reactions in the wall of the

nasopharynx. It has been repeatedly shown that tonsils are composed of T and B cells. The latter are capable of synthesizing all the major immunoglobulin classes (18, 30), and specific antibodies (2, 16). Specific cell-mediated immune responses of tonsillar cells were demonstrated in some studies (10, 16, 17, 26).

The present study was undertaken to compare immunological functions of the pharyngeal tonsil, palatine tonsils and blood

of children undergoing tonsillectomy. This was performed by determining the E or EAC binding lymphocytes, responses to mitogens and cell-mediated immunological reactions to tuberculin. The migration ability of tonsillar cells and their usefulness in direct migration inhibition assay were also determined.

MATERIAL AND METHODS

Preparation of cells from blood and tonsils

Specimens of peripheral blood and tonsils, collected from 2- to 12-year-old children undergoing tonsillectomy and adenoidectomy, were evaluated. Blood samples (about 20 ml) were obtained from 21 children immediately before tonsillectomy. Palatine tonsils from 31 and pharyngeal tonsils from nine children were placed in the sterile cold medium 199 (Institute of Immunology, Zagreb) immediately after tonsillectomy and processed within 30 min.

Cell suspensions

The buffy coat was separated from heparinized blood (25 IU heparin/ml) by spontaneous sedimentation and divided into two aliquots. One was used for the separation of mononuclear cells on bovine serum albumin gradient as described by Dekaris et al. (3). The mononuclear cells were used for the rosette assay. Leucocytes from the other part were used in lymphocyte transformation and leucocyte migration tests. The isolated cells were washed three times in medium 199 before testing.

Tonsillar lymphocytes were obtained by gentle grinding of tonsillar tissue fragments over a sterile wire mesh (100 mesh, Cadish & Sons, London) with medium 199. The cell suspension was washed three times in the same medium and the cell pellet was finally resuspended in 5 ml of the medium. The number of cells was adjusted to a final concentration after the counting in a hemocytometer. About 90% of the cells were

found to be viable by trypan blue-dye exclusion test.

Rosette assay

For the E- and EAC-rosette assays with lymphocytes from the peripheral blood and tonsils, we used the method of Holm et al. (9). Lymphocytes bearing four or more erythrocytes were considered positive and the results were expressed as a percentage.

Lymphocyte transformation

Cell suspensions were adjusted to a concentration of 2.4×10^6 viable nucleated cells per tube containing 3 ml of medium 199 supplemented with 15% human AB serum. The following optimum doses of mitogens or of tuberculin (22) were added to each of the triplicate cell cultures: PHA (Wellcome, Beckenham, Kent) 90 µg, Con A (Sigma Chemical Comp., St. Louis) 150 µg, and PPD (RT 32, Statens Serum Institut, Copenhagen) 100 µg/ml. Triplicate cultures in medium alone were used as controls. The cultures were incubated for 96 h at 37°C, the cells were harvested, and red cells removed by osmotic shock. The cell suspension was introduced in vaseline chambers, and permanent preparations were made by fixation with 2.5% glutaraldehyde, as described by Silobrić et al. (29). At least 100 cells from each preparation were counted and a number of blast-like cells determined under a phase-contrast microscope (Orthoplan, Leitz, magnification $\times 650$). The degree of transformation was evaluated as a percentage of blast-like cells.

Migration inhibition assay

The migration of leucocytes from the peripheral blood and cells from tonsillar tissue was assessed in the presence of tuberculin according to the technique proposed by Federlin et al. (5), slightly modified by Silobrić et al. (29). The macrophage migration inhibition assay ("two-step" technique) was performed by the

method of Rajapakse & Glynn (23), with minor modifications. The capillaries were filled with cell suspension (70×10^6 cells/ml) containing 75% guinea pig macrophages and 25% lymphocytes from tonsillar tissue. In all cases the PPD concentration was $100 \mu\text{g/ml}$ suspension. Two to three capillaries were set in a migration chamber filled with either medium 199 enriched with 10% horse serum (control), or the same medium containing $100 \mu\text{g}$ of tuberculin/ml (experimental). After 2 and 24 h of incubation at 37°C , the migration area was projected onto paper through a photographic enlarger, drawn, cut and weighed. The area of migration was given in square centimeters of paper covered with a 10 times enlarged projection of the actual migration area. Migration inhibition indices were calculated as (the area of migration in the presence of antigen/the area of migration in control medium) $\times 100$.

RESULTS

T and B cells

Using the rosette technique we determined the percentages of T and B cells in tonsillar tissue and peripheral blood samples. The percentage of T (E-rosette) and B (EAC-rosette) cells in palatine or pharyngeal tonsils and peripheral blood of 16 children is shown in Table 1. In the peripheral blood the mean percentage of E-rosettes was significantly higher and that of EAC-rosettes lower, than in the palatine or pharyngeal tonsils. There was no difference in the mean number of E- or EAC-rosettes in palatine or pharyngeal tonsils.

The same proportions of E- and EAC-rosettes are attained if only complete series of paralleled tests (children Nos. 4, 6, 7, 8, 9, and 10) were evaluated, or if all results

Table 1
Percentages of E- and EAC-rosettes in peripheral blood and tonsillar tissue

Child No.	Peripheral blood		Palatine tonsils		Pharyngeal tonsils	
	E	EAC	E	EAC	E	EAC
1	—	—	—	—	36.0	48.0
2	—	—	38.6	31.2	—	—
3	48.1	43.2	24.9	31.2	—	—
4	49.4	34.2	30.4	45.3	39.7	56.2
5	—	—	32.4	45.8	—	—
6	56.9	40.1	43.2	38.8	45.3	38.2
7	53.8	24.8	38.1	39.1	29.4	35.8
8	60.6	14.5	25.1	29.4	43.0	28.0
9	51.5	18.2	30.3	35.0	47.0	44.0
10	58.6	24.3	23.4	48.1	27.1	41.8
11	—	—	30.0	39.0	—	—
12	—	—	46.4	46.8	—	—
13	47.5	14.4	46.1	24.5	—	—
14	—	—	43.7	46.2	—	—
15	—	—	34.8	58.4	—	—
16	—	—	42.9	42.6	—	—
n	8	8	15	15	7	7
Mean $\pm$ s.d.	53.3 ± 4.9	26.7 ± 11.2	35.3 ± 7.9	40.0 ± 8.8	38.2 ± 4.5	41.7 ± 9.0
P ^a			0.001	0.01	0.001	0.01

^a P has been calculated by the Student's *t*-test. Significance of the difference between mean E- and EAC-values in the peripheral blood versus palatine or pharyngeal tonsils was calculated. There were no differences between the mean E- and EAC-values in palatine and pharyngeal tonsils.

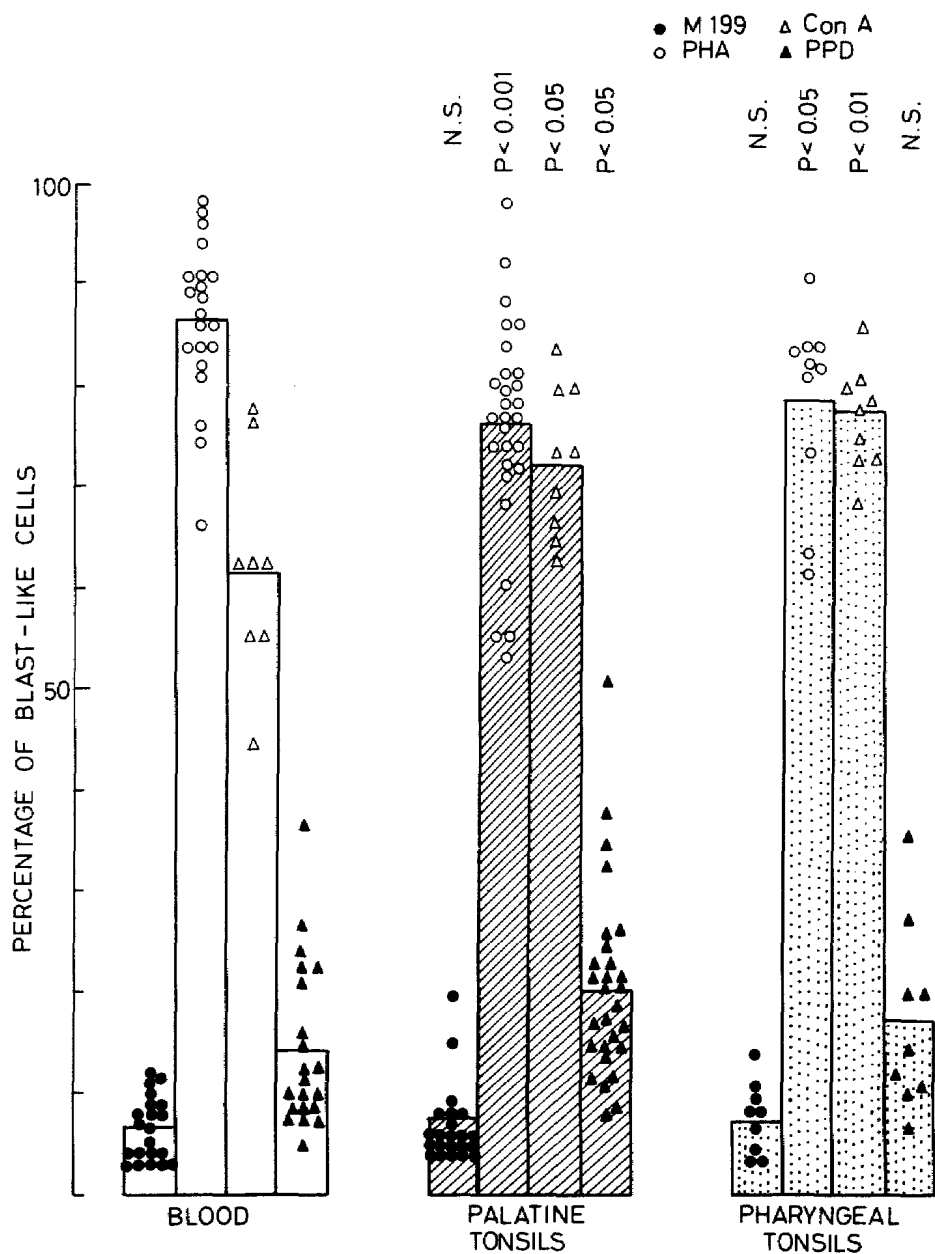


Fig. 1. The percentage of blast-like cells in a 96-h culture of cells from palatine or pharyngeal tonsils and peripheral blood. These cells were cultivated in medium alone (●) or in the same medium containing 90 μ g PHA (○), 150 μ g Con A (Δ), or 100 μ g PPD (▲) per ml. Each individual result is represented, while the top of the column corresponds to the mean value for the group. Different levels of significance (*P*, Student's *t* test) for the difference between the results obtained with blood leucocytes and tonsillar cells are indicated.

presented in Table 1 were taken into account.

Lymphocyte transformation

This technique was used to determine the lymphocyte transformation in cultures with T mitogens and tuberculin. Fig. 1 presents lymphocyte transformation expressed as the mean percentage of blast-like cells in cultures from palatine or pharyngeal tonsils and peripheral blood leucocytes. It appears that in the presence of PHA there were, on an average, significantly fewer blast-like cells in tonsillar cell cultures (both tonsils) than in blood leucocyte culture. Conversely, in the parallel cultures run with Con A the average number of blast-like cells was significantly higher in tonsillar cultures than in blood leucocyte cultures. In the presence of PHA or Con A there was no difference in the average number of blast-like cells in cell cultures derived from palatine and pharyngeal tonsils respectively.

In all cultures containing tuberculin there

were significantly higher percentages of blast-like cells than in those with medium alone. Further, the transformation in palatine tonsillar cell cultures was more pronounced than in the corresponding blood cell cultures. Again, there was no difference in lymphocyte transformation between the cultures containing palatine or pharyngeal tonsil cells.

Migration inhibition assay and migration ability of tonsillar cells

This testing was performed in order to determine the ability of tonsillar lymphocytes to produce migration inhibitory factor (24) in the presence of tuberculin and also to check whether these cells alone could be used for direct migration inhibition testing.

The migration of the mixture containing human tonsillar cells and guinea pig peritoneal cells was determined in chambers containing medium alone or the same medium supplemented with tuberculin. As shown in Table 2, migration indices at 2 or

Table 2

Migration indices in medium containing 100 µg of PPD/ml at 2 and 24 h. Cells from palatine tonsils were assayed by direct test (D) and two-step test (TS)

Child No.	2 Hours		24 Hours	
	D	TS	D	TS
1	70 ^a	77 ^a	66 ^a	62 ^a
2	82	75 ^a	80	66 ^{a, b}
3	63 ^a	64 ^a	72 ^a	86 ^b
4	95	98	106	101
5	89	93	98	96
6	96	105	88	92
7	83	106 ^b	74 ^a	76 ^a
8	63 ^a	73 ^{a, b}	80	73 ^a
9	81 ^a	82	78 ^a	75 ^a
10	76 ^a	74 ^a	78 ^a	70 ^a
11	72 ^a	64 ^a	81	75 ^a
12	109	94 ^b	92	81 ^b
13	89	99 ^b	98	97
14	93	93	93	104 ^b
Mean ± s.d.	83 ± 13.3	86 ± 14.5	85 ± 11.5	82 ± 13.6

^a Significant migration inhibition (see text).

^b A greater than 10% difference in indices obtained by two-step or direct migration inhibition technique.

24 h intervals were distributed above and under the index of 81 and 79 respectively. Previously, by an analysis of Mantoux positive and Mantoux negative children, these indices were shown to represent the highest index still showing a significant degree of migration inhibition of blood leucocytes cultivated with 100 µg of tuberculin per ml (28). According to this parameter, six and seven out of 14 children, at 2 and 24 h respectively, exhibited significant migration inhibition. Indices obtained by a parallel testing of tonsillar cells were quite similarly distributed and there was no difference in the mean inhibition indices obtained by the two-step or by the direct migration inhibition techniques. If the indices produced by these two tests are analysed for each child, a difference greater than 10% is found in four out of 14 parallel testings. Thereafter, nearly identical results are produced by two-step or by the direct migration inhibition techniques. This was

unexpected, since tonsillar tissue is composed predominantly of non-migrating lymphoid cells (38). However, as the direct test using tonsillar cells is much simpler than the two-step technique, the subsequent experiments were performed only using the direct technique. By parallel testing of blood leucocytes and cells from palatine and pharyngeal tonsils we obtained almost identical mean migration inhibition indices at 2- or 24-h intervals (Table 3).

We have studied the migration ability of tonsillar cells. As shown in Table 4, cells from palatine and pharyngeal tonsils are migrating, albeit poorly, and they cover significantly smaller migration areas than blood leucocytes of the same patients.

DISCUSSION

Studies of immunological functions of tonsillar cells could provide data on the characteristics and importance of local

Table 3

Comparison between mean migration indices of peripheral blood leucocytes, palatine and pharyngeal tonsillar cells incubated for 2 and 24 h in medium containing 100 µg of PPD/ml

Incubation time (hours)	Peripheral blood		Migration indices for cells from:					
			Palatine tonsils			Pharyngeal tonsils		
	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>P</i> ^b	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>P</i> ^b
2	22	83 ± 19	24	79 ± 13	>0.05	9	82 ± 11	>0.05
24	22	90 ± 11	22	91 ± 9	>0.05	9	96 ± 9	>0.05

^a Number of tested persons.

^b Not significant as compared to the peripheral blood cells by Student's *t*-test.

Table 4

Mean migration areas of palatine and pharyngeal tonsillar and peripheral blood cells incubated for 2 h and 24 h in medium 199 alone

Incubation time (hours)	Peripheral blood		Migration areas (cm ²) for cells from:					
			Palatine tonsils			Pharyngeal tonsils		
	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>P</i> ^b	<i>n</i> ^a	$\bar{x} \pm \text{s.d.}$	<i>P</i> ^b
2	20	6.0 ± 1.5	25	2.3 ± 0.8	<0.001	9	2.3 ± 0.6	<0.001
24	20	32.5 ± 8.3	23	17.5 ± 3.6	<0.001	8	15.0 ± 4.0	<0.001

^a Number of tested persons.

^b The significance of the difference between values in peripheral blood and palatine or pharyngeal tonsils respectively, was calculated by the Student's *t*-test.

immunity in the nasopharynx and clarify the role of tonsils in the pathogenesis of some diseases as well as in the protection of the local mucosal system. Our aim has been to study differences between systemic and local immunity and to compare the responses of cells derived from palatine and pharyngeal tonsils. The study has revealed differences in the proportion of T and B cells and in their responses in tonsils, as compared to the peripheral blood lymphocytes, and a similarity between those parameters, determined for the cells from palatine and pharyngeal tonsils. Since our assays were performed simultaneously on blood and tonsillar cells from the same child, the results were not influenced by the known individual variations in the proportions of T and B lymphocytes (31) and *in vitro* tests of cell-mediated immune responses (1, 15).

Over 90% of cells from the mechanically disaggregated palatine tonsils are lymphocytes and blasts (38). The proportion of these cells binds sheep erythrocytes (8, 10, 26, 32, 34, 38) and is equivalent to a T cell (11). The remaining population of tonsillar cells bind erythrocytes sensitized by antibody and complement (8, 32) and correspond to a B cell (27). The percentage of T cells revealed by E-rosetting varies from 23% (32) to 59% (8), whilst about 39% tonsillar cells were shown to form EAC-rosettes (8, 32). Tonsillar T cells were found in peri- and interfollicular zones and B cells in germinal centres (33). Our results are in agreement with some of those previously reported. Similarly to Hurtado et al. (10) and Willson et al. (38) we have found that 35% cells of palatine tonsils are T cells. Others have found (8, 32), as we did, that about 40% of tonsillar cells are B cells. Further, we have shown, similarly to Gupta et al. (8), a lower proportion of T cells in tonsils, as compared to blood lymphocytes. In other studies, this difference in the proportion of T and B cells in tonsils and blood was demonstrable only for T, but not for B cells (32), or was not statistically significant for T cells (10). Differences in results in various reports could

possibly be explained by age-dependent changes in the proportions of T and B lymphocytes in children (6, 7) and by the suppressive effect of respiratory infections on the T cell population (14).

The reactions of tonsillar lymphocytes to mitogens have been studied *in vitro* by an increasing number of authors. Significant responses to PHA (4, 10, 16, 17, 20, 26, 34, 38), Con A and PWM (26, 38) have been detected either by ³H-thymidine incorporation (4, 10, 16, 17, 26, 34, 38) or by morphologic evaluation (17, 20, 34). In this study, we have evaluated the magnitude of transformation through analysis in a phase contrast microscope. This method was previously successfully used for the detection of transformed blood lymphocytes cultivated with various mitogens and antigens (22). In this study, contrary to Oettgen et al. (20), we have not observed a higher number of blast-like cells in control cultures containing tonsillar cells, as compared to the cultures of circulating lymphocytes, but in accordance with others (17, 20, 34) we have found a significant response of tonsillar cells to the stimulation with PHA and Con A. The reactions of cells from palatine tonsils and the pharyngeal tonsil were identical, but the reactions of all the tonsillar cells differed from the responses of the circulating lymphocytes. In accordance with Hurtado et al. (10), the response to PHA in our study was higher in blood lymphocytes than in tonsillar cells. We have shown that reverse responses, i.e. higher response of tonsillar cells, were caused by Con A. It is difficult to interpret these findings, but they clearly show the particularity of the responses of tonsillar lymphocytes as compared to reactions of blood lymphocytes. At least one fact should be mentioned. The proportion of B cells is higher in tonsils than in blood, and PHA, albeit considered as T cell mitogen (19), transforms human B cells also (21).

The role of secretory immunoglobulins occurring locally at mucous membrane sites has been extensively investigated (35). Much less is known about the local cell-mediated

immunity (CMI) in the mucosal system. There is evidence that in animals the respiratory tract is independent of the systemic CMI (36, 37, 39). In humans, specific local CMI of tonsillar cells (10, 16, 17, 20, 26) and cells obtained by bronchoalveolar lavage (12) was *in vitro* tested by lymphocyte transformation (16, 17, 20, 26), macrophage migration inhibition (12, 16) and lymphocytotoxicity tests (10). These earlier studies demonstrated CMI to rubella vaccine (10, 16, 17), streptolysin S, diphtheria, polio, pertussis, tetanus antigens (20), PPD (12, 26), *Candida* (26), influenza and mumps virus antigens (12). We have compared the responses of tonsillar and blood lymphocytes to PPD. Tuberculin seemed suitable for the following reasons: tuberculin allergy is the classical example of cell-mediated immune response; protection against *M. tuberculosis* entering through the respiratory tract is more closely related to local CMI of this tract than to systemic CMI (39), and being BCG-vaccinated immediately after birth, nearly all children in our country are Mantoux-positive. The results of our study provide evidence for positive CMI to tuberculin both in tonsils and blood. However, whilst in migration inhibition test the tonsillar lymphocytes exhibited responses similar to those seen in circulating lymphocytes, lymphoproliferative responses were higher in cultures with tonsillar lymphocytes. It is a known fact that results produced by these two *in vitro* correlates of CMI are not always identical (13, 25), although the correlation between migration inhibition and lymphocyte transformation assays was once reported for tonsillar cells cultured in the presence of rubella virus antigen (16). In our study the cells from the palatine and pharyngeal tonsil reacted similarly to PPD, exhibiting local CMI to tuberculin, which was either equal to (in migration inhibition test) or higher than (in lymphocyte transformation test) systemic CMI.

The finding that tonsillar cells alone are usable for migration inhibition tests seems to

be of interest. As tonsils are composed predominantly of lymphocytes with very few polymorphs, monocytes or macrophages (38), tonsillar cells seemed unsuitable for the direct migration inhibition testing. Thus like Morag et al. (16), we started our work by mixing tonsillar cells with guinea pig peritoneal cells. Then we performed parallel testing of cell-mediated immunity to tuberculin of mixed tonsillar and guinea pig cells, and tonsillar cells alone. Unexpectedly, we got very similar responses. This indicates that a simple direct technique using tonsillar cells alone, could be successfully used for migration inhibition studies of tonsillar cells. Later, we studied the migration ability of tonsillar cells. They migrated considerably slower if compared to the blood leucocytes of the same persons, but migration inhibition caused by tuberculin was nearly identical in cultures containing either tonsillar cells alone, or blood leucocytes.

In conclusion, our findings support and extend the evidence on particularity of tonsillar lymphocytes and their cell-mediated immune responses, and demonstrate the usability of tonsillar cells in direct migration inhibition testing.

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