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# Study of human T and B lymphocytes with heterologous antisera

## III. IMMUNOFLUORESCENCE STUDIES ON TONSIL SECTIONS

J.-P. LAMELIN,* NICOLE THOMASSET,*§ C. ANDRE,† J. BROCHIER‡ & J.-P. REVILLARD‡ *International Agency for Research on Cancer, 150 cours Albert Thomas 69008 Lyon †INSERM U45, Hôpital Edouard Herriot, Place d'Arsonval, 69008 Lyon and ‡INSERM U80, Department of Immunology, Université Claude Bernard, Lyon

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**Summary.** Lymphoid cells from human palatine tonsils were identified on tissue sections by membrane or intracellular immunofluorescence (IF) staining. Used in an indirect technique, an anti-IgM and an anti-HTLA (human T lymphocyte antigen) antiserum gave complementary patterns of membrane IF, characteristic of the follicular organization. When serial sections were stained for each of the five classes, immunoglobulin-containing cells from all classes were found. Their relative frequencies were, in decreasing order: IgG: 61·6%; IgA: 17·3%; IgM: 12%; IgE: 7·5%; IgD: 1·6%. These differed from those of the gut-associated lymphoid tissue (IgA>IgG>IgM) and of the peripheral lymph nodes (IgG>IgM>IgA), but were close to those of mesenteric nodes. The absence of secretory component in tonsil was an additional difference from gut-associated lymphoid tissue and the relatively high proportion of IgE-containing cells, in the absence of recognized atopy, is another feature in common with mesenteric lymph nodes. Finally, slight differences between these results and those obtained on tonsil cell suspensions suggest that some degree of selection probably occurs during the isolation procedure.

§ Present address: INSERM U51, 1, Place Joseph-Renaud, 69008 Lyon, France.

Correspondence: Dr J.-P. Lamelin, Unit of Biological Carcinogenesis, Centre International de Recherche sur le Cancer, 150 Cours Albert-Thomas, 69372, Lyon Cedex 2, France.

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## INTRODUCTION

Most of the techniques (e.g. neonatal thymectomy, chronic administration of anti-lymphocyte serum) used in animals for localizing thymus-dependent and -independent areas in lymphoid organs (Cooper, Peterson & Good, 1965; Gowans & McGregor, 1965) are not suitable for routine investigation in man. This requires a direct approach, which consists in identifying cells in tissue sections, by appropriate surface markers. Initially developed in animals (Gutman & Weisman, 1972), this technique was then successfuly applied to man (e.g. Whiteside, 1977). With tonsils, however, no data were available until the recent contribution, based upon immunoenzyme staining, of Curran & Jones (1977).

In the present paper, we took advantage of the heterologous anti-human T-lymphocyte antigen (HTLA) antiserum previously described (Brochier, Abou-Hamed, Gueho & Revillard, 1976) to investigate, by indirect immunofluorescence (I.F.) the structure of 'normal' human tonsils.

## MATERIALS AND METHODS

*Tonsil specimens*

These were obtained from five patients aged 3–26 years undergoing surgical removal for recurrent tonsillitis.

*Specific antisera*

The anti-HTLA antiserum was raised in horse and made specific as previously described (Brochier *et al.*, 1976). Rabbit anti-human immunoglobulin G, M, A, D and E antisera were assessed for specificity by immunoelectrophoresis against plasma proteins. All these reagents were purchased (Behringwerke) as fluorescein conjugates. One of them (anti-IgM) was also obtained unconjugated for use in membrane staining by the indirect IF technique. A rabbit anti-human secretory component without activity against lactoferrin, was kindly provided by Dr S. Baudner (Marburg-Lahn, Germany). All reagents were freed of aggregates by high speed centrifugation (150,000 *g* for 60 min).

*Tissue processing*

*For membrane immunofluorescence.* Fresh tonsils were cut into small pieces (2–3 mm), snap-frozen in liquid nitrogen, and kept at $-70°$ until use. Cryostat sections (4 $\mu$m) were fixed either in ethanol (1 min at $-20°$) or in phosphate buffered saline (PBS) 0·2% solution of paraformaldehyde (10 min at 0°), washed in PBS and thereafter never allowed to dry.

*For intracellular staining.* Fragments of tonsils were fixed in 96% ethanol (24–48 h., 4°) either directly or following 24 h incubation in cold PBS. This usually decreased the background by washing off the interstitial proteins (Brandtzaeg, 1974). 4 $\mu$m serial sections were cut after paraffin embedding.

*Immunofluorescent technology*

*Membrane staining.* The indirect IF technique required the use of fluorescein (Fl)-coupled anti-rabbit and anti-horse immunoglobulins. In view of the high background observed with two commercial batches of Fl-anti-rabbit immunoglobulin (Ig) we prepared our own conjugates. The immunoglobulin fractions of a sheep anti-rabbit Ig and a rabbit anti-horse Ig were prepared by DEAE cellulose chromatography, absorbed on tonsil cells and polymerized human Ig, before being coupled to fluorescein isothiocyanate (FITC, BBL, Cockeysville, Md.) under alkaline conditions (Cebra & Goldstein, 1965). Following separation of free FITC by passage through a Sephadex G25 column, conjugates with homogeneous fluorescein/protein (F/P) molar ratios were selected by stepwise gradient chromatography on DEAE cellulose (phosphate buffer 0·025 M pH 7·3 containing NaCl at increasing concentrations). Suitable reagents (F/P molar ratio: 2 to 3) were usually obtained between 0·1 M and 0·3 M NaCl.

The most reproducible and best results were obtained after ethanol fixation for the detection of surface Ig, and after paraformaldehyde fixation for HTLA positive cell identification (See discussion). The staining was done in two steps. The unconjugated specific antiserum, diluted in Hanks solution containing 5% bovine serum albumin (BSA, Difco Labs Inc., Detroit, Mich., 30% BSA stock solution, 5% BSA—Hanks), was first layered on the tissue section, and left standing for 20 min at room temperature. Following washing in PBS, the sections were then covered for 15 min with the corresponding fluorescein conjugate also diluted in 5% BSA—Hanks. Following washing in PBS and mounting in glycerol:PBS, (9:1), the slides were examined under phase contrast (for evaluating the tissue preservation) and U.V. light with a Leitz Orthoplan photomicroscope equipped with a Ploem illuminator. Pictures were taken on high speed Tungsten Ektachrome.

*Intracellular staining* was done in one step, on adjacent sections, each being stained for one of the five immunoglobulin classes. Differential counts were established by enumerating the brightly-stained class-specific plasma cells on the whole surface of the sections.

## RESULTS

**Thymus-dependent and -independent areas**

As shown on Fig. 1a the staining by anti-IgM antibodies was restricted to the lymphoid follicle. Few plasma cells were detected. The area made up of small lymphocytes surrounding the follicle was not stained and was therefore likely to correspond to the T-dependent area. This was confirmed when the adjacent section (Fig. 1b) was stained with anti-HTLA, and gave a fluorescence pattern complemen-

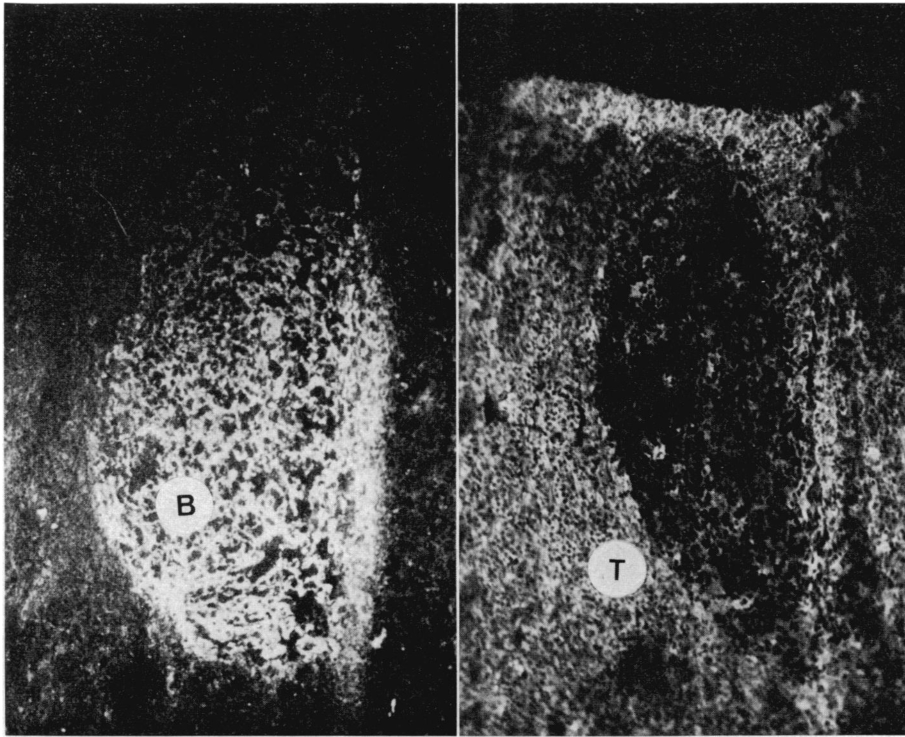


**Figure 1.** Indirect immunofluorescence staining of T-dependent (T) and -independent (B) areas on adjacent sections of tonsil: (a) lymphoid follicle stained with an anti-IgM antibody; (b) HTLA positive cells surrounding a lymphoid follicle (dark area) scattered with a few bright cells. This pattern of staining is complementary of that seen on (a). (× 100)

**Table 1.** Relative proportions of Ig-containing cells of different heavy chain specificities in tonsils*

| Tonsil no. | Class specificity | | | | |
|---|---|---|---|---|---|
| | IgA | IgM | IgG | IgD | IgE |
| 1 | 1121† (30·5) | 433 (11·7) | 1823 (49·6) | 12 (3) | 285 (8) |
| 2 | 32 (5) | 89 (13·5) | 490 (74) | 27 (4) | 22 (3·5) |
| 3 | 150 (15) | 93 (9) | 545 (54) | 117 (12) | 99 (10) |
| 4 | 800 (14) | 643 (11·5) | 3795 (67) | 42 (1) | 370 (6·5) |
| 5 | 669 (13·5) | 650 (13) | 3200 (64) | 65 (1) | 421 (8·5) |
| Total number | 2772 | 1908 | 9853 | 263 | 1197 |
| Percentages | 17·3 | 12·0 | 61·6 | 1·6 | 7·5 |

* Established on a series of adjacent sections, stained with the five heavy chain specific antisera. For each class, the whole surface of an identical number of sections was examined.

† Actual number recorded. Percentage between parentheses.

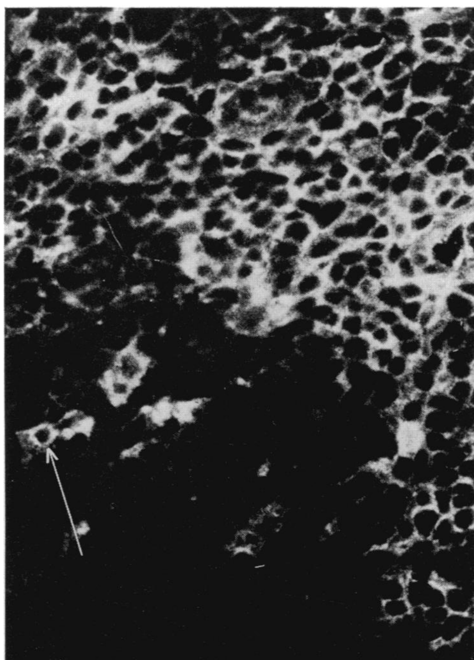

**Figure 2.** Details of the thymus-dependent (fluorescent) and -independent (dark with a few scattered bright cells) areas. Most of the cells are easily identified as HTLA positive or negative. The arrow shows one of the large intrafollicular HTLA positive cells (X 400).

tary to that revealed by the anti-IgM antibody. In the early stages of these experiments, a rabbit anti-light chain antiserum was also used. The staining pattern was similar, but the background was consistently higher with a faint intracellular staining of numerous Ig producing cells scattered across the section.

At higher magnification (Fig. 2) a clear cut distinction could be made between positively and negatively stained cells (thus allowing the identification of individual T cells). The scattering of rare HTLA positive cells inside the lymphoid follicle is noteworthy (arrow, Fig. 2).

**Localization and class distribution of immunoglobulin-containing cells**

IgG and IgA specific plasma cells were unequally distributed with a higher density around the follicles (Fig. 3) and in the vicinity of the epithelium including that lining the crypts. Inside the follicles a few cells characteristically formed a ring around the germinal centre (Fig. 4). No special pattern could be ascribed to the localization of the much scarcer IgM, D and E containing cells.

When the relative numbers of Ig-containing cells were established according to their class, on serial sections stained each for a given isotype, the mean percentages were 61·6% IgG, 17·3% IgA, 12·0% IgM, 7·5% IgE and 1·6% IgD (Table 1). None of the specific antibodies, including that

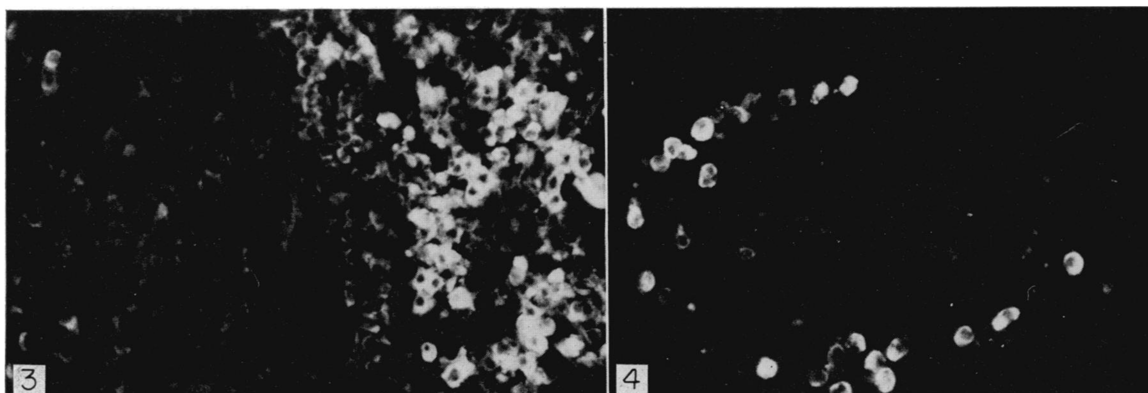


**Figure 3.** IgG containing cells surrounding a lymphoid follicle (dark area on the left). (× 250)
**Figure 4.** Ring of IgA containing cells, inside a lymphoid follicle surrounding a germinal centre. (× 250)

directed against the IgA secretory component, gave any staining of the epithelial cells.

**Role of fixation and fluorescein conjugates**

The high background observed after acetone and the autofluorescence resulting from glutaraldehyde fixation (2% in cacodylate buffer 0·1 M pH 7·4 or 0·5% in association with paraformaldehyde 1% in $PO_4$ buffer 0·1 M pH 7·3) made these fixatives unsuitable for our study.

In the indirect immunofluorescence technique, the use of conjugates with an homogeneous F/P ratio was critical in decreasing the non-specific background.

## DISCUSSION

It has already been suggested that the structural organization of tonsils is closer to that of lymph nodes than to that of the gut-associated lymphoid tissue: lymphoid follicles centered by a germinal centre and surrounded by numerous plasma cells are separated from each other by a zone of small lymphocytes infiltrated with Ig producing cells. The T-dependence of the inter-follicular areas has been postulated (Curran & Jones, 1977).

We have presented evidence that the great majority of inter-follicular cells are indeed thymus-derived by indirect IF using an anti-HTLA reagent, the specificity of which has previously been reported (Brochier *et al.*, 1976). As the cells were identified in sections, we had to first examine whether the reagent kept its specificity under these conditions. Greater sensitivity or the detection of structures absent from circulating cells, might have obscured the picture.

That the staining with anti-HTLA was essentially restricted to T cells was controlled by identifying T-independent areas on adjacent sections. For this purpose, an anti-Ig antibody was preferred to the anti-HMBLA antibody because the latter reacts with both cells and monocytes (Brochier *et al.*, 1976). An anti-IgM specific antibody, rather than a polyspecific anti-Ig, was selected since, besides the wide acceptance of membrane-bound IgM as a marker of human B lymphocytes (Seligmann, Preud'Homme & Brouet, 1973) little interference from the fluorescence of IgM plasma cells was likely to occur, their contribution to their total number being close to 10%. Both reagents were used as intact molecules, since the scarcity of Fc receptors bearing cells in tonsils (Samarut, Brochier & Revillard, 1976) makes these an unlikely source of confusion.

The finding of a few HTLA positive cells scattered inside the lymphoid follicles is noteworthy. Unstained and control sections (on which normal horse serum replaced anti-HTLA) confirmed that this staining was specific, although the cells were larger than those stained in the interfollicular areas, and their staining more diffuse. Despite this morphological difference, those cells may still belong to T cell lineage. Alternatively they may have a different origin, the staining being due either to an antigenic structure common to T and non-T cells, or to some contaminant antibody recognizing a non T-cell structure. Similar unexplained observations have been made, in animal (Gutman & Weissman, 1972) as well as in other human lymphoid organs (Schoorl, Brutel de la Rivière, von dem Borne & Feltkamp-Vroom, 1976).

During the set up of the indirect IF for membrane staining on tissue sections, several technical points were found to be critical. Not all fixatives were appropriate, and moreover, when two different structures were identified, optimal results (good tissue preservation, sharp contrast between stained and unstained areas) were obtained with different fixation procedures. Paraformaldehyde, unsuitable for Ig staining in our hands as in Curran and Jones' experience (1977), was the best for T cell identification. The reverse was true for ethanol. Similar empirical observations have been made for other structures studied by IF on tissue sections (Fairfax, Doniach & Wells, 1976; Goldschneider, Gregoire, Barton & Bollum, 1977). This might well prevent the successful staining on the same section of two non-cross reactive structures using different fluorochromes.

Following our initial observation that commercial fluorescent reagents (at least two of them) gave a a high fluorescent background, we coupled our antisera and selected by stepwise elution, conjugates with a F/P molar ratio of 2 to 3, this mean value corresponding to a restricted range of coupling ratios. Although their mean F/P ratio may be similar, unselected reagents are more heterogenous, with unlabelled molecules competing for the specific antigenic structures and overconjugated material responsible for non-specific staining.

Immunoglobulin-containing cells of all classes were detected in all our five patients. This is in agreement with the data of Curran and Jones (1977) using an immunoperoxidase technique. We suggest that the observation by Ostergaard (1975) of an absence of IgE and IgA plasma cells in 60 and 30% of his patients respectively, is due to technical reasons. By using unfixed tissue, the penetration inside the cells of the fluorescein conjugates was obviously difficult and might have been the cause with two of them for negative results. This interpretation is supported by the author's own comment that in one patient it was not possible to find fluorescing plasma cells at all, in spite of the unmistakable, although sparse, occurrence of plasma cells detected by methyl pyronin green staining.

The relative frequencies of the various classes ($\gamma>\alpha>\mu>\varepsilon>\delta$) differed from those observed in peripheral lymph nodes: $\gamma>\mu>\alpha$ (Mellors & Korngold, 1963) with $\varepsilon<1\%$ (Tomasi, 1976) and negligible $\delta$ (Burtin, Guilbert & Buffe, 1966). It also differed from those recorded in human gut-associated lymphoid tissue: 76% $\alpha$, 12% $\gamma$, 9% $\mu$, 2% $\varepsilon$, 1% $\delta$ (C. André, unpublished data) but was comparable to the distribution in mesenteric lymph nodes: 56% $\gamma$, 24% $\alpha$, 8% $\mu$, 7% $\varepsilon$ and 5% $\delta$ (C. Andrè, unpublished data).

Our five patients underwent surgery some time after an acute infection and, although their IgE serum level was not determined, none had a clinical history of atopy. The percentages here, including the 7·5% IgE, are therefore likely to reflect a 'normal' pattern of distribution.

As to the preferential localization of IgG and IgA containing cells, an observation also made by Curran & Jones (1977), it might correspond to some intimate relationship with other cell types. The simultaneous use of two fluorochromes would be of great interest: the lack of a common fixation procedure for membrane and intracellular staining, at least on tissue section, has so far made this technique unapplicable.

Finally, two observations suggest that the method of preparing cell suspensions in our own and other studies (e.g. Willson, Luberoff, Pitts & Pretlow II, 1975; Gergely, Vanky & Klein, 1976) might have introduced some bias in the recovery of the various cell populations: firstly the distribution of the different class specific immunoglobulin producers established on tonsil sections (Table 1) is different from that calculated on cell suspensions (Brochier Samarut, Lethibichthuy & Revillard (in press), and, secondly, examination of many serial sections strongly suggests that the T-dependent territory, and, therefore, probably their absolute cell number, is larger than its T-independent counterpart. This possibility, already discussed on a functional basis by Willson, Zaremba, Pitts & Prelow II (1976), is presently under investigation.

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