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Immunoglobulin-containing cells in human tonsils as demonstrated by immunohistochemistry

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SUMMARY

Intracellular immunoglobulin has been demonstrated in human palatine tonsils by the unlabelled antibody peroxidase–antiperoxidase complex (PAP) method in which rabbit antiserum to a range of human immunoglobulins (Igs) was linked to the PAP complex by an intermediate stage of swine antiserum to rabbit Ig. The effects of different methods of fixation and processing have been compared, formol-saline fixation giving the best results. The PAP technique proved greatly superior to the fluorescein isothiocyanate (FITC)-based technique, not only in sensitivity but in permitting study of the finer histological and cytological features.

The lymphoid follicles are shown to have three distinct zones, two forming the follicle centre (zones (a) and (b)), and the third (zone (c)) the lymphocyte cap. Ig synthesis appeared to begin in the cells in zone (b). IgG, IgA, IgM, IgE and IgD were present in all tonsils, with IgG predominating, confirming that the tonsil resembles lymph nodes more closely than it does alimentary lymphoid tissue. Some follicles contained more than one type of Ig. The tonsil appears to have a well-developed T-dependent area, the lymphoid follicles forming a B-cell area.

The structure of the tonsil would seem to facilitate contact between its lymphoid tissue and antigens in the crypts, and it is postulated that some T cells within the crypt epithelium, after contact with antigen, may leave the tonsil by the efferent lymphatics and enter the peripheral circulation by the thoracic duct, whilst other primed T cells interact with B cells in the follicle centres. Some B cells may then start to synthesize immunoglobulin, whilst others become memory cells in the lymphocyte 'cap' of the follicle.

INTRODUCTION

Previous studies using fluorescein isothiocyanate (FITC)-labelled antisera have shown the presence in human tonsils of immunoglobulin (Ig) of various types, located both intracellularly and extracellularly. Because of the limitations of fluorescence microscopy, however, the identity of the cells containing Ig could not be determined with certainty (Crabbé & Heremans, 1967). Peroxidase-linked antibody techniques offer considerable advantages over immunofluorescent methods (Petts & Roitt, 1971), in allowing detailed study of the tissues by conventional light microscopy. Better methods of fixation and processing of the tissues can also be employed, along with a range of other histochemical techniques. It was decided therefore to apply the immunoperoxidase technique to human tonsils, in order to determine the distribution of Ig and Ig-containing cells in a way not possible by fluorescence microscopy. By utilizing a variety of other histochemical and histological techniques it was hoped to throw some light on the immunological functions of the tonsil.

MATERIALS AND METHODS

Twenty-seven palatine tonsils, removed surgically from patients whose ages ranged from 7–36 years, were cut into slices

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(1–2 mm thick) with a new degreased razor blade. From these, paraffin and cryostat sections were prepared by a variety of procedures.

Paraffin sections. Initially several different fixatives were used with each tonsil. The tissues were gently agitated during fixation (except in method 4).

(1) (a) Four per cent formaldehyde in phosphate buffer pH 7 (0·135 M Sorensen), using paraformaldehyde (Maunsbach, 1966); (b) 4% formaldehyde in phosphate buffer; (c) 4% formaldehyde in physiological (0·85%) saline (formol-saline); (d) 4% aqueous formaldehyde solution. Occasionally sucrose (1%, 3% or 5%) was added to the fixation solution and sometimes Tris buffer (0·2 M) was used instead of phosphate buffer. The period of fixation in formalin was generally 24 hr at 20°C but some tissues were also fixed for 24 hr at 4°C. The most consistent results were obtained after fixation for 24 hr at 20°C in formol saline and this was later used routinely. After fixation the tissues were processed through 74 OP ethanol (8 hr) and chloroform (5 hr) to paraffin wax (5 hr at 56°C).

(2) Glutaraldehyde/formalin/calcium acetate solution (Garvin, Spicer & McKeever, 1974) for 2 or 24 hr at 4° or 20°C, followed by embedding in paraffin wax as in (1).

(3) Ninety-five per cent ethanol for 24 hr at 4°C followed by embedding in paraffin wax as described by Sainte-Marie (1962).

(4) Diethylpyrocarbonate (DEPC). Thin (2 mm) slices of tissue were immersed for 10–15 sec in isopentane cooled with liquid nitrogen and then transferred, using chilled forceps, to a Pearse tissue dryer (Edwards High Vacuum) for drying under vacuum at −60°C for 18 hr. The dried tissues were fixed in DEPC vapour for 3 hr at 55°C in a sealed container (Pearse *et al.*, 1974) and embedded for 1 hr under vacuum in paraffin wax in an Autotechnicon Ultra.

Cryostat sections. Blocks of tissue, immersed in Tissue-Tek OCT compound and quenched in isopentane, were used for the preparation of cryostat sections (6–8 μm). The sections were cut at −20°C, mounted on albumen-coated slides, air-dried, and post-fixed in formol-saline (15 min), alcohol (5 min) or glutaraldehyde (5 min). Cryostat sections were also prepared from tissues which had been fixed in formol-saline (5 or 24 hr at 20°C) and then quenched in isopentane.

Staining methods. Sections were stained by Harris's haematoxylin and eosin; Gordon & Sweets' method for reticulin; Grocott's method (Grocott, 1955) for fungi; Gram's method for micro-organisms; Marshall's metalophil impregnation method (Marshall, 1948); and by the Unna-Pappenheim sequence for ribonucleic acid. Mast cells were identified by means of polychrome methylene blue.

Immunohistochemical procedure. Ig's were demonstrated in the tissues by the unlabelled antibody peroxidase-antiperoxidase complex (PAP) method, in which rabbit antiserum to human Ig is linked to the PAP immune complex by an intermediate stage of swine antiserum to rabbit Ig. The swine antiserum attaches to the rabbit Ig (specifically bound to the Ig in the tissue section) as well as to the rabbit Ig in the PAP complex. The sites of binding of the PAP complex are revealed by means of 3,3-diaminobenzidine tetrahydrochloride (DAB).

After treatment with xylene and ethanol (74 OP), paraffin sections (5 μm) were placed on a rocker tray (Denley Instruments) and treated as follows at room temperature (20°C): (a) 0·2% HCl in methanol (Mazurkiewicz & Nakane 1972): 30 min. This step blocked endogenous peroxidase activity (in erythrocytes, eosinophil leucocytes and mast cells) in formalin-fixed tissues but failed with tissues fixed in glutaraldehyde. It was superior to the methanol/hydrogen peroxidase sequence (Streefkerk, 1972); and the α-naphthol/pyronin sequence (Taylor & Burns, 1974) gave 'false-positive' staining of plasma cells by the pyronin. (b) Rinse in water, followed by Tris-saline buffer (15 vol 0·1 N HCl, 10 vol. 0·2 M Tris, 15 volume normal saline). (c) Normal (non-immune, non-conjugated) swine serum 1 in 5 in Tris-saline buffer: 30 min. The serum was tipped off without rinsing in buffer. This step is meant to reduce non-specific binding of the rabbit anti-human Ig antisera (Burns, 1975). (d) Rabbit anti-human Ig antiserum 1 in 160: 30 min. The antiserum in this stage and in stages (b) and (h) were diluted in Tris-saline buffer. Although this was a useful titre for routine use, the optimum titre varied from specimen to specimen, and a range of titres was generally performed for each specimen to find the optimum, which ranged from 1 in 40–1 in 680. Positive results were sometimes obtainable with titres as high as 1:8000, particularly with DEPC-fixed tissues. Lowering of the titre invariably tends to induce or increase non-specific reactions. It does not, however, increase the strength of specific reactions, and cells reacting strongly at high titres may react weakly at lower titres. Antisera* were used against the following: IgG, IgG Fc fragment (IgG (Fc)), IgA, IgA secretory component (lacking antibodies to α chains) (IgA (SC)), IgM, IgD, IgE and kappa (K) and lambda (L) chains. (e) Wash, with stirring, in buffer: 30 min. (f) Swine anti-rabbit Ig antiserum 1 in 20: 30 min. (g) Wash in buffer: 30 min. (h) PAP complex† 1 in 40: 30 min. (i) Wash in buffer 20 min. (j) Cover with freshly-made 0·05% DAB-peroxidase solution (5 mg DAB in 10 ml Tris-saline buffer and 1 drop (0·025 ml) of 30% (100 vol.) H_2O_2): 5 min. (k) Wash in water and counterstain (1 min) with Mayer's haemalum. (l) Dehydrate, clear and mount in Canada balsam.

Cryostat sections were treated in the same way as paraffin sections except for an additional step, between stages (i) and (j), of fixation (15 min in 4% formol-saline or 5 min in 1·5% glutaraldehyde in S-collidine buffer).

Controls. Sections were reacted with DAB solution alone, to reveal sites of endogenous peroxidase activity. Other sections were reacted with the full sequence of reactions except for the omission of stage (d), (f) or (h). Normal (non-immune)

* The IgD and IgE antisera were obtained from Behring (Hoechst) and the other sera (code numbers 10–090, 10-MAT, 10–091, 10–OOG, 10, MSP, 10–9K2, 10–9L2 and 21–090) from Dakopatts A/S of Copenhagen, Denmark (through Mercia Diagnostics Ltd, Watford).

† Dakopatts Code Number Z113.

rabbit serum was used in stage (d) in place of rabbit anti-human Ig and at the same titre, to help detect non-specific reactions. The use of a number of different antisera in stage (d) also provides effective control of specificity, in that each antiserum reacted with a different population of cells, the number of which varied with the particular antiserum used. Sites which gave a reaction with all the antisera used in stage (d) and with normal rabbit serum were regarded as reacting non-specifically.

FITC method. A two-stage sandwich method using FITC-labelled antiserum (swine anti-rabbit Ig after stage (d)) was tried in the early stages of the study but it proved less sensitive and so much less convenient than the PAP sequence that it was discontinued.

RESULTS

The palatine tonsil consists of lymphoid tissue deeply penetrated by epithelium-lined crypts which communicate with the oro-pharynx and often contain desquamated epithelial cells, sometimes in large numbers. The other cells present include a high proportion of lymphocytes. Filamentous and coccal micro-organisms are occasionally seen, but the numbers are comparatively small and many crypts contain none. None of the tonsils shows evidence of acute inflammation or suppuration.

Epithelium

Each crypt is lined by stratified squamous epithelium and is surrounded by a sheath of lymphoid tissue, with lymphoid follicles located at regular intervals immediately beneath the epithelium (Fig. 1). The epithelium rarely shows keratinization and lacks a well-defined and continuous basement membrane. It is markedly reticular and the spaces between the cords of epithelial cells are filled with lymphocytes and plasma cells (Figs 1 and 2). The reticular structure is particularly pronounced in the smallest crypts which penetrate almost to the capsule of the tonsil and have little or no lumen. In the more superficial layers of the epithelium the predominant cells are Ig-negative small lymphocytes and only an occasional Ig-containing cell is found. The lymphocytes are separated from the lumen by only one or two epithelial cells and many penetrate this barrier to reach the crypt. In the basal layers of the epithelium the number of lymphocytes is sometimes so great that it is difficult to distinguish the lower epithelial border. There are also many Ig-containing cells in this zone and just beneath the epithelium (Fig. 3). They are mature plasma cells, with intense cytoplasmic pyroninophilia. The concentration of Ig in these cells is considerable and sometimes it appears greater than in similar cells more superficially placed in the epithelium.

The epithelium on the pharyngeal aspect of the tonsil lacks the reticular structure of crypt epithelium and has a well-formed basal layer of columnar cells. These differences may account for the fact that it contains fewer lymphocytes than the crypt epithelium and these are invariably confined to the basal layers. No Ig-positive cells are present.

Follicles

All the follicles have germinal centres. A well-orientated section, at right angles to the surface of the crypt epithelium and passing through the centre of a follicle, shows that the follicle has 'polarity' and can be divided into three zones, each with a morphologically distinct population of cells; two zones ((a) and (b)) form the centre of the follicle and the third (c) is the mass of small lymphocytes 'capping' and enclosing the centre (Fig. 1). The upper limits of zone (c) are often indistinct, from the presence of many lymphocytes in the epithelium. Polarity is also evident in a reticulin preparation, in which the top of the follicle (i.e. the part nearer the crypt) appears relatively 'open' whereas the base of the follicle rests on a compact layer of fibrils (Fig. 4).

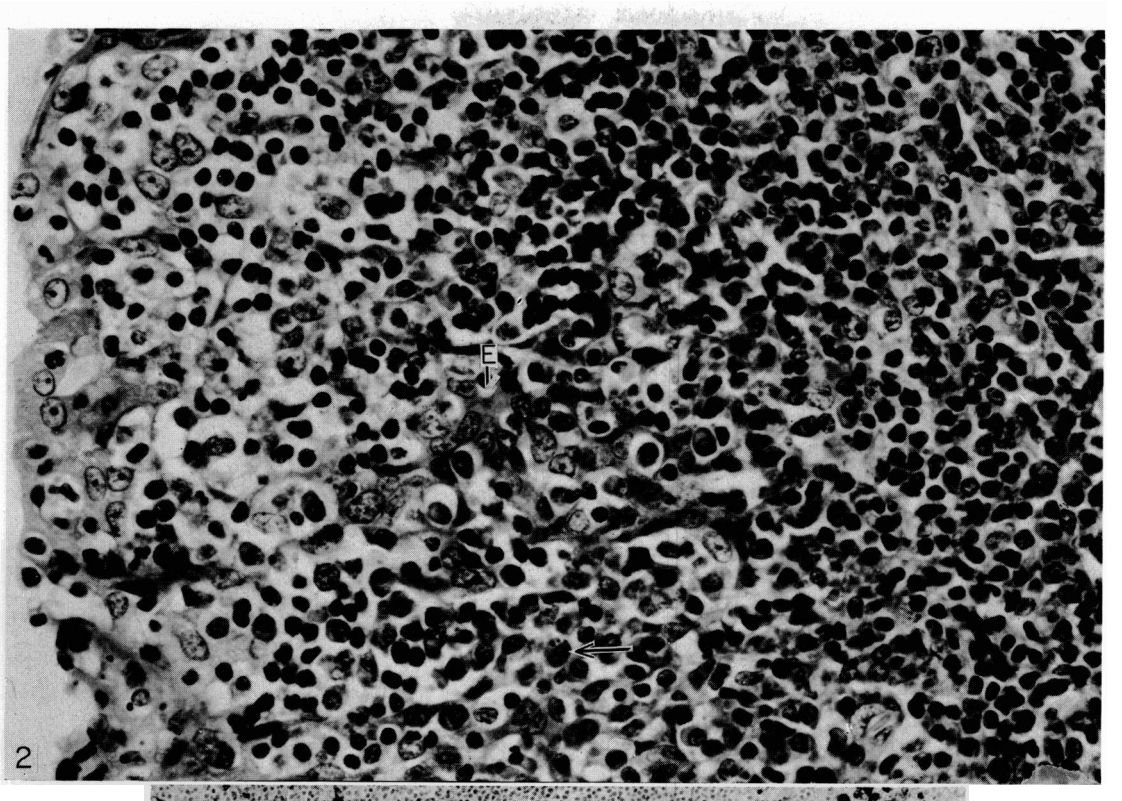
Zone (a). This is the basal part of the follicle centre i.e. the part distal from the crypt. The cells have large, vesicular and moderately basophilic nuclei, many of which are in mitosis (Fig. 1). The amount of cytoplasm is relatively small but in most cells it exhibits pyroninophilia. A few cells may contain Ig, in small amounts. Large round macrophages with abundant cytoplasm are present. Their cytoplasm often contains 'tingible bodies' but no Ig. In hyperplastic follicles, zone (a) extends to occupy more of the follicle centre. The number of macrophages is correspondingly increased but their regular pattern of distribution is retained, giving the follicle centre a 'starry-sky' appearance (Fig. 5).

Zone (b). This is the part of the follicle centre lying nearer the crypt (Fig. 1). The cell nuclei are less basophilic but more pleomorphic than those in zone (a) and mitotic figures are infrequent. The cells

have more cytoplasm than those in zone (a) but in only a small proportion is it pyroninophilic. When pyroninophilia is present, however, it is similar in intensity to that exhibited by mature plasma cells, except that it is often focal and not diffuse. A considerable number of cells contain Ig (Figs 6 and 7) and they tend to be located towards the periphery of the zone (Fig. 8). In well-fixed tissues the Ig in many cells consists of a small dense mass adjacent to the nucleus. Although this region of the cell corresponds to the juxtanuclear 'halo' of plasma cells, very few of the Ig-containing cells have the nuclear structure of mature plasma cells. Sometimes IgG-containing cells are present in practically all the follicle centres; and in the same follicles there are frequently cells containing other types of Ig, most often IgA.

FIG. 1. Crypt epithelium (E) overlying a lymphoid follicle with a well-developed germinal centre. The closely-packed cells in the bottom third of the follicle centre (zone (a)) have large vesicular nuclei and little cytoplasm. Mitoses (arrow) are numerous, and large macrophages (M) containing chromatin fragments (tingible bodies) are present. The cells in zone (b) (top two-thirds of the follicle centre) have more cytoplasm and the nuclei are more pleomorphic. Small lymphocytes form a 'cap' (zone (c)) over the follicle centre. A few histiocytes (H) are visible in this zone. Lymphocytes are also present in considerable numbers within the epithelium. PCV = post-capillary venule. (H & E magnification × 192)

Histiocytes are present throughout the zone. Although smaller than the macrophages of zone (a), they are highly dendritic by the metalophil method. The cells nearer the crypt are more dendritic than those deeper in zone (b) and there is a gradual transition from the large round non-dendritic or poorly-dendritic macrophages of zone (a) to the highly dendritic cells in the more superficial part of zone (b).



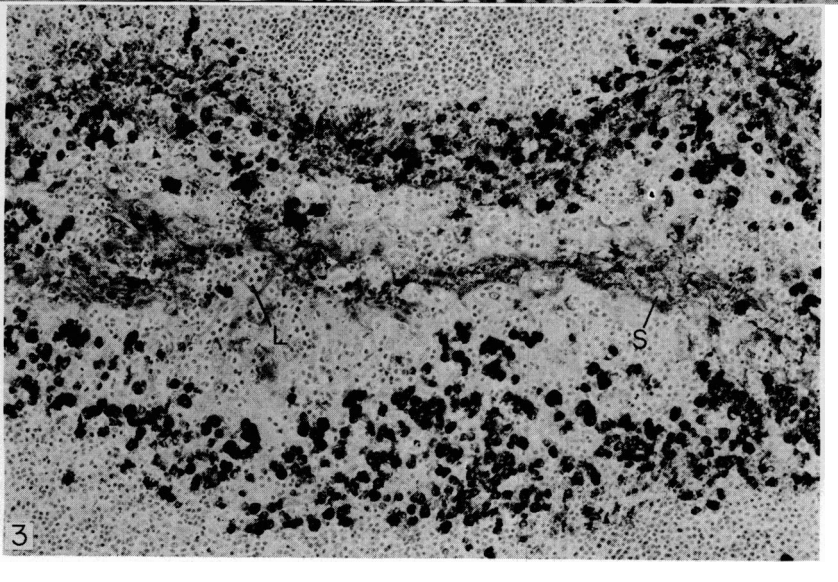
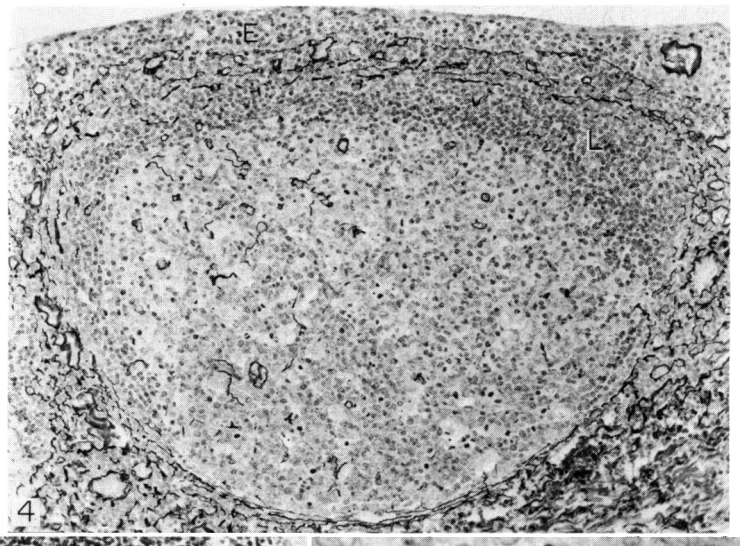
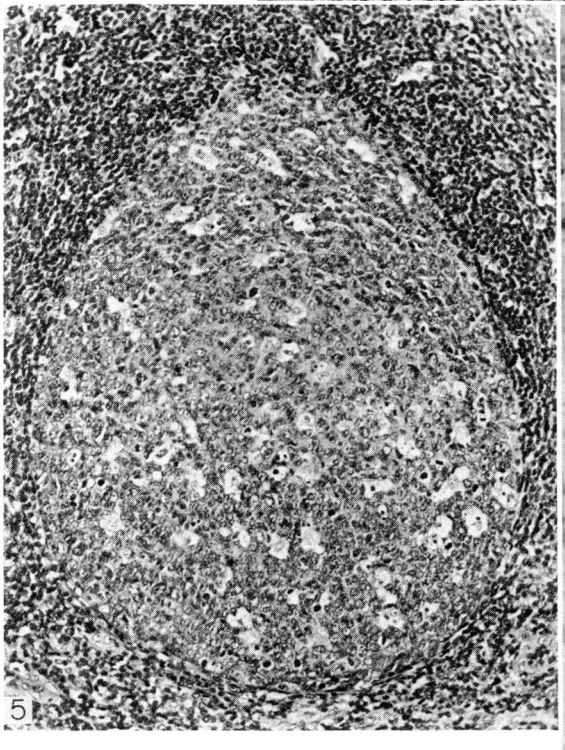


FIG. 2. Crypt epithelium showing many small lymphocytes and occasional plasma cells (arrow) lying between the cords of epithelium cells (E). There are also many lymphocytes in the sub-epithelial tissues (right), so that the lower border of the epithelium is obscured. (H & E Magnification × 468.)

FIG. 3. Small crypt in cross-section. The lumen is detectable by the presence of squames (S) giving a non-specific reaction. Large numbers of IgG-containing cells (black) are present in and beneath the epithelium The small cells in the more superficial parts of the epithelium are lymphocytes (L). PAP method for IgG (Magnification × 198.)





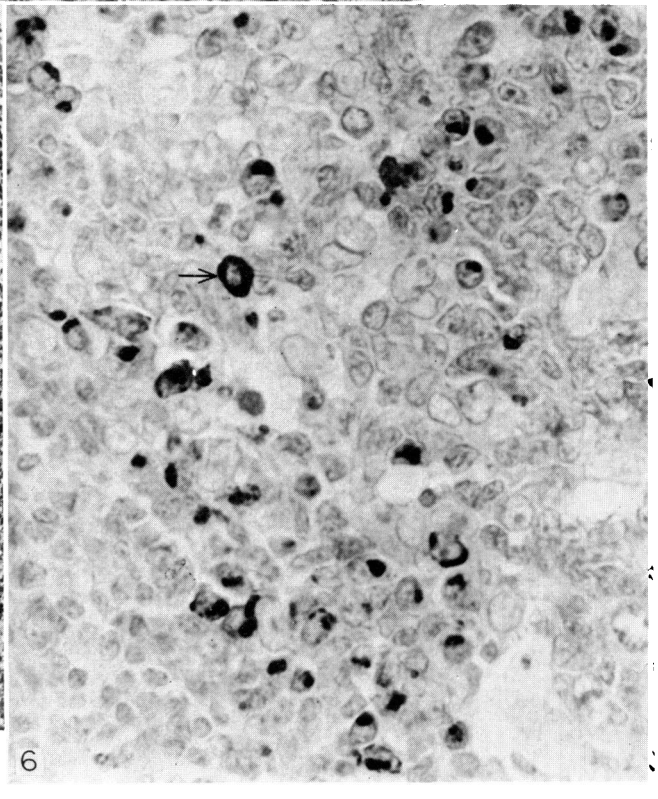


FIG. 4. Lymphoid follicle and crypt epithelium (E), showing reticulin fibrils. Above the follicle the fibrils are discontinuous but beneath it they are continuous and several layers thick. The circular fibrils, some inside the follicle centre, are the basement membranes of small blood vessels, including post-capillary venules at the sides of the follicle. L = lymphocytes of zone (c). Gordon & Sweets' method. (Magnification × 126.)

FIG. 5. Hyperplastic lymphoid follicle. Zone (a) forms most of the enlarged follicle centre, and the number of large macrophages, many containing nuclear fragments, has increased proportionately. A few histiocytes are also present among the small lymphocytes of zone (c) (top). (H & E Magnification × 126.)

FIG. 6. Part of zone (b) of a follicle centre. Many cells contain small amounts of IgG (black), located near the nucleus of the cell. In one cell (arrow) it is present throughout the cytoplasm. PAP method for IgG. (Magnification × 468.)

Zone (*c*). This part of the follicle is a crescentic 'cap', covering zones (a) and (b), of small cells with the densely basophilic nuclei and very scanty cytoplasm of small lymphocytes (Fig. 1). No mitoses are detectable among these cells. A few dendritic histiocytes are usually present.

Other lymphoid tissue

The tissues around the follicles are rich in small lymphocytes, and Ig-containing cells similar to those in the epithelium are present in moderate numbers. Sometimes a row of flattened Ig-containing cells lies between the lymphoid tissue and the base of each follicle (Fig. 7). A few mast cells are seen and post-capillary venules (PCV) are plentiful in this part of the tonsil (Fig. 9), which is presumably a

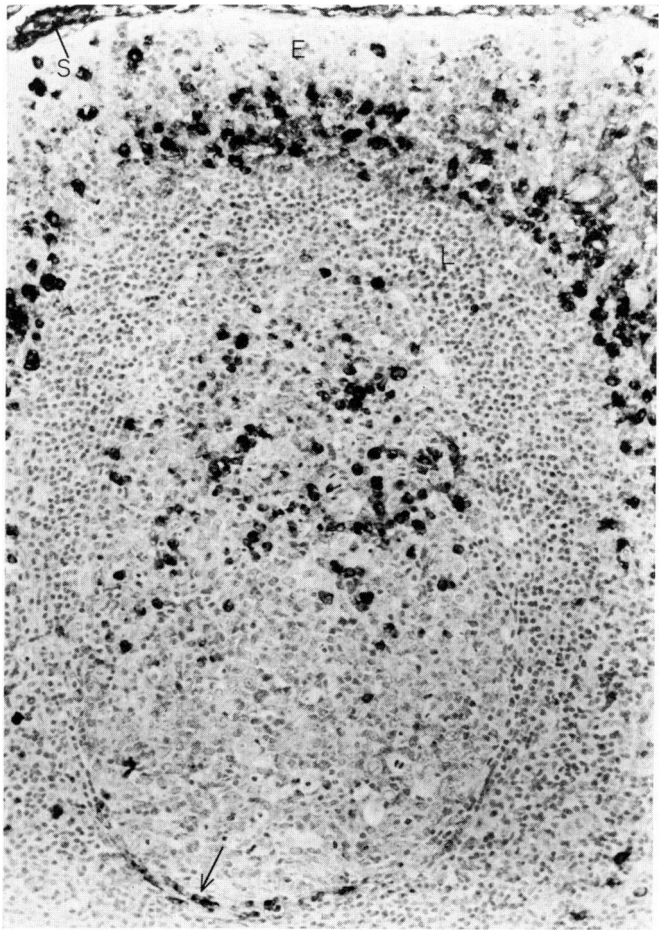
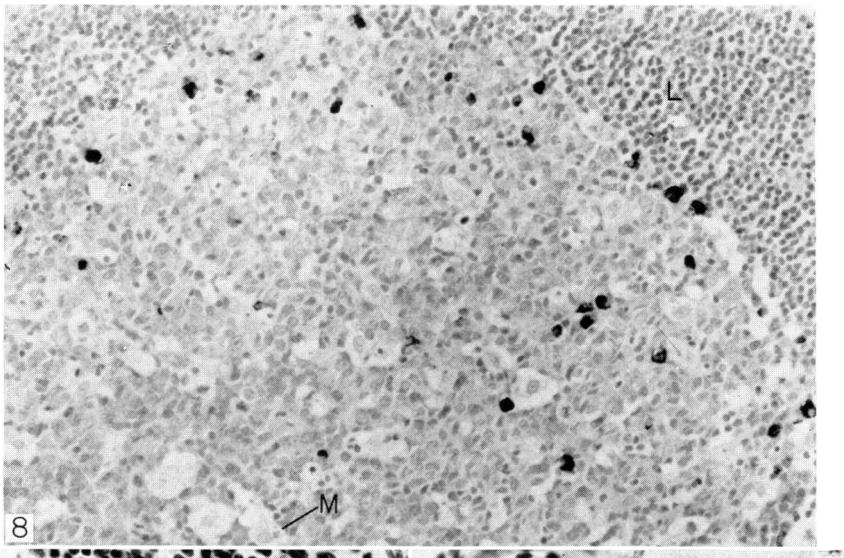


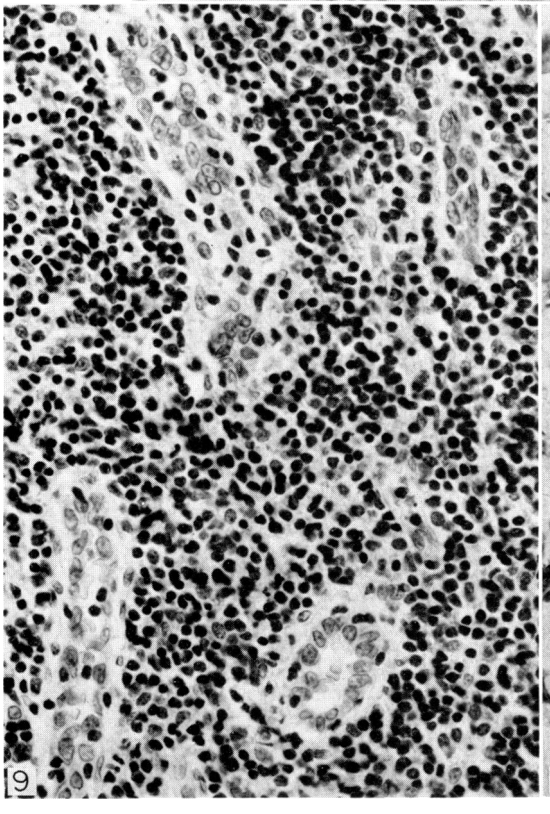
FIG. 7. Lymphoid follicle and crypt epithelium (E), showing many cells containing IgG (black) in and beneath the epithelium, as well as in zone (b) of the follicle centre. A few flattened cells beneath the follicle also contain IgG (arrow). Squames on the surface of the epithelium (S) give a non-specific reaction. L = lymphocytes of zone (c). PAP method for IgG. (Magnification × 180.)

thymus-dependent area. The PCV are lined by very large endothelial cells and small lymphocytes are present in the lumen and in the walls of the vessels. There is a plexus of thin-walled capillary blood vessels beneath the crypt epithelium, and many capillaries actually lie within the epithelium.

Capsule and stroma

The tonsil is enclosed in a fibrous tissue capsule, and trabeculae of fibrous tissue intersect the organ to form a network upon which the follicles and associated lymphoid tissues rest. The stromal connective





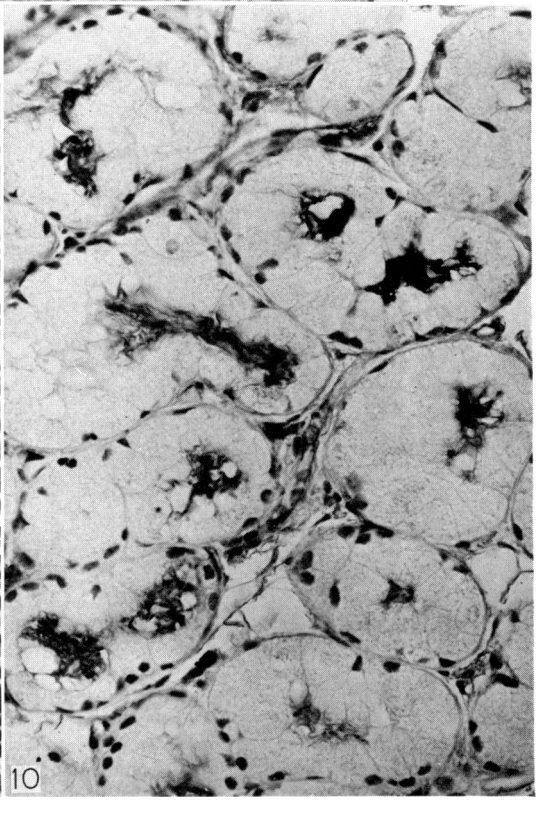


FIG. 8. Lymphoid follicle, showing IgM-containing cells (black) in zone (b). In some cells the amount of IgM is small. M = macrophage in zone (a). L = lymphocytes of zone (c). PAP method for IgM. (Magnification × 246.)

FIG. 9. Peri-follicular lymphoid tissue showing four post-capillary venules, lined by large endothelial cells. Small lymphocytes are present in the lumen and in the vessel walls. (H & E Magnification × 324.)

FIG. 10. Mucous gland. IgA (black) is present in the mucus secretion in all the acini. PAP method for IgA. (Magnification × 324.)

tissue is vascular and considerable numbers of Ig-containing cells are often present. Mast cells are also fairly numerous, tending to congregate round the blood vessels.

Types of Ig and their frequency distribution

Igs G, A, M, D and E are present intracellularly in all the tonsils, and a consistent pattern is observed with regard to the numbers of cells containing the various types of Ig: IgG-positive cells are the most numerous and the next most numerous are IgA-positive cells. Considerably fewer IgM-positive cells are found and these show a tendency to be located in zone (b) (Fig. 8). Cells containing IgE and IgD are also present in all tonsils in relatively small numbers. No association can be detected between the IgE-positive cells and mast cells. The distribution of positively-reacting cells is usually not uniform throughout the tonsil, but by using serial sections (one section for each type of Ig) and counting the positively-reacting cells in a rectangular area which includes one follicle and the overlying epithelium, it is possible to obtain an approximate estimate of the frequency distribution (Table 1). Absolute numbers of cells are not recorded, the frequency distribution being determined on a six-point scale whereby 0 = no cells; ± = occasional cells present; + = 1–25 cells; ++ = 26–50 cells; +++ = 51–100 cells and ++++ = greater than 100 cells present. This estimate agrees with subjective assessments of the frequency distribution for the whole tonsil.

Occasional IgG- and IgA-containing cells are found in zone (a) but IgM, IgE and IgD are very infrequent. The most active part of the follicle centre is zone (b) where up to fifty IgG-positive cells can usually be identified and up to twenty-five cells positive for IgA, IgM, IgE and IgD, in decreasing

TABLE 1. Frequency distribution of Ig-containing cells within defined area

	Follicle			
Ig class	Zone a	Zone b	Zone c	Remainder of area
IgG	±	++	±	++++
IgG(Fc)	±	++	±	++++
IgA	±	+	±	++
IgM	±	+	±	+
IgE	±	+	±	+
IgD	±	+	±	+
IgA(SC)	0	0	0	0

0 = Nil; ± = cells present occasionally; + = 1–25 cells; ++ = 26–50 cells; +++ = 51–100 cells; ++++ = >100 cells.

order of frequency. In the lymphocyte cap (zone (c)) an occasional cell positive for IgG or IgA may be present. The largest concentration of intracellular Ig is found in the basal layers of the epithelium and just beneath it, and to a lesser extent around the lateral margins of the follicle. The positive cells are frequently so numerous in this region, especially IgG-containing cells, that it is often only possible to estimate their number as in excess of 100 cells within the defined area. IgA-containing cells are about half as numerous as the IgG cells, followed by IgM, IgE and IgD in decreasing order.

Effects of fixation and processing

Tissues fixed in paraformaldehyde give results consistently inferior to those fixed in formol saline or aqueous formalin. The addition of sucrose to the fixative is always deleterious: the plasma cells appear shrunken, any positive reaction seems to be confined to the cell surface, and no intracellular reaction is seen. The results with cold ethanol fixation are inconsistent. Sometimes strongly positive reactions are obtained but with a few exceptions preservation of cell structure is not good and it is difficult to localize activity to individual cells. Fixation in glutaraldehyde-containing solutions gives the best histological preservation of cell structure, and the reaction product for Ig is often located precisely to the region adjacent to the cell nucleus. However, fixation for more than a few hours in glutaraldehyde weakens the immunohistochemical reaction, and tissues fixed in it for 18–24 hr are usually negative.

Freeze-dried DEPC-fixed tissues give the strongest reactions and the Ig-positive cells appear larger than in tissues processed in other ways, with good preservation of nuclear and cytoplasmic structure. Cryostat sections of quenched tissue, post-fixed in formol-saline, also react to a high titre, with good preservation of cell structure, but further fixation before DAB is needed to prevent diffusion of the reaction product and to preserve the tissue structure from the destructive effects of the DAB reaction. Cryostat sections prepared from tissue fixed for 24 hr in formol-saline prior to quenching react fairly well, with good cellular preservation, but in those pre-fixed for only 5 hr vacuolation is so pronounced as to destroy the finer histological structure.

Some cryostat sections were unexpectely negative. It was found that the albumen coating on the slides on which the sections had been lifted had not been completely dry when the sections had been applied. The albumen had presumably diffused into the sections and caused complete blocking of both specific and non-specific reactions.

Non-specific reactions

Non-specific reactions appear stronger in tissues fixed in alcohol or glutaraldehyde and may persist in tissues which are 'over-fixed' and negative for Ig. They are given by all the antisera used in stage (d), including anti-IgA(SC) and particularly by normal (non-immune) rabbit serum. The sites include fibrous tissue (but not dense collagen); fibrin; tonofibrils and sometimes individual epithelial cells (generally large and vacuolated), as well as squames lying in the crypts; and occasionally zone (b) of the follicle centre. Cell cytoplasm may also give a non-specific reaction when the titre for stage (d) is too low. This tendency is particularly marked with mature plasma cells, especially when normal rabbit serum is used in stage (d). When the optimum titre is used for stage (d), non-specific reactions are absent or relatively weak. The application of normal swine serum (stage (c)) reduces the intensity of non-specific reactions but the strength of the specific reactions is equally reduced.

Endogenous peroxidase

The use of HCl/methanol (stage (a)) effectively blocks endogenous peroxidase activity in tissues fixed in formalin but inconsistently in those adequately fixed in glutaraldehyde.

Mucous gland

Mucus-secreting salivary gland tissue is present on the external aspect of the fibrous capsule of most of the tonsils examined. The stroma of the gland usually contains small numbers of plasma cells, most of which are IgA-positive. A few react for IgG and an occasional one is IgM-positive. Frequently some of the acinar cells and the mucinous secretion within the small ducts are positive for IgA and IgA(SC) (Fig. 10). Less often these sites also contain IgG.

DISCUSSION

The tonsil has no afferent lymphatics, the crypt system obviously performing the same function of bringing antigens into contact with the lymphoid tissues. This and a number of other structural features suggest that the tonsil plays an important role in exposing the host to a wide range of environmental antigens at an early stage of extra-uterine development and in facilitating the host's response to these antigens: the free communication between the crypts and the oro-pharynx, allowing micro-organisms to enter and lodge there; the extensive surface area of the epithelial lining of the crypts; the reticular structure of the crypt epithelium, housing a large population of lymphocytes and plasma cells; the apparent ease with which lymphocytes penetrate the epithelium to reach the crypts; and the location of large numbers of lymphoid follicles immediately beneath the epithelium.

For these reasons it might be expected that a wide range of Igs would be synthesized within the tonsil, and this was confirmed by the presence of Igs G, A, M, D and E in all the tonsils examined. The predominance of IgG-positive cells also showed that the lymphoid tissue of the human tonsil is closer immunologically to lymph nodes and spleen than to the lymphoid tissues associated with the alimentary mucosa, in which IgA-positive cells greatly outnumber the other types (Crabbé, Carbonara & Heremans, 1965). The absence of IgA (SC) in the tonsils is also noteworthy.

Two features of crypt epithelium distinguish it from other epithelia: the presence within it of a plexus of thin-walled capillaries; and the reticular structure. The latter is so pronounced as to suggest that the epithelium functions less as a barrier between the lymphoid tissues of the tonsil and the crypt than as a network able to contain large numbers of lymphocytes and plasma cells, perhaps for considerable periods of time. The morphological resemblance between tonsillar epithelium and thymic epithelium is obvious but whether the former has functional or secretory activities comparable to those attributed to thymic epithelium is unknown.

The lymphocytes within the crypt epithelium are presumably T cells which have entered the tonsil via the post-capillary venules in the peri-follicular tissues; and those located near the surface of the crypt epithelium are well placed to make contact with antigenic materials in the crypts. Some of the lymphocytes are known to enter the crypt, to be excreted as 'salivary corpuscles'. Others no doubt leave the tonsil by the efferent lymphatics which drain into the jugulo-digastric lymph node in the deep cervical chain and thence into the thoracic duct. The return of primed lymphocytes to the circulating pool via the thoracic duct would represent peripheral sensitization.

Other T lymphocytes sensitized at the epithelial surface might interact with the cells in the follicle centre, which are a sub-population of B lymphocytes (Craddock, Longmire & McMillan, 1971), either by migrating there or by secreting a diffusible factor (Geha *et al.*, 1973). The B cells could respond in two ways. Firstly, some of them might divide and become large lymphocytes (immunoblasts) which begin to synthesize Ig and eventually become plasma cells. Secondly, other B cells might undergo mitosis and migrate into the lymphocyte 'cap' (zone (c)), to constitute a pool of 'memory' cells, there being good evidence that these memory cells originate in the follicle centre (Koburg, 1967). Cells leaving zone (c) and entering the epithelium would, on meeting the appropriate antigen, undergo a secondary response and form Ig.

We have no evidence as to the fate or turnover rate of the many Ig-containing cells, histologically mature plasma cells, in and beneath the epithelium. On a few occasions the amount of Ig seemed to be less in the cells nearer the lumen than in those located more deeply, suggesting that the cells moved towards the lumen and lost Ig as they did so, but this was so infrequent as to make this unlikely. On the other hand the marked tendency for the highest concentration of Ig-containing cells to be found in the basal layers of the epithelium suggests that the population of cells here is relatively static.

The reactions for various Igs in the follicle centre were focal, intense and intracellular, whereas previous reports, based on the use of fluorescent antibodies, tended more to show a diffuse reaction, e.g. for IgG, IgA and IgM (Crabbé & Heremans, 1967; Østergaard, 1975) as well as for IgE (Tada & Ishizaka, 1970). It has been suggested that the cells within the follicle centre constitute a single clone (Burtin & Buffe, 1967) and that each germinal centre produces antibody of only one specificity (White, 1958). Our finding of more than one type of Ig within a follicle centre does not exclude the possibility of the Igs being homogeneous with regard to antigenic specificity, although it does show that they are heterogeneous with regard to molecular class. Ig has been demonstrated on the surface of dendritic histiocytes in the follicle centre (Balfour & Humphrey, 1967; White, French & Stark, 1970) but nothing resembling this pattern was seen in the present study. The antibody on the surfaces of the dendritic histiocytes is thought to bind antigen from antigen-bearing lymphocytes (Mitchell & Abbot, 1971) and subsequently present it to B lymphocytes (Nossal, Abbot & Mitchell, 1968); and it may be significant that dendritic histiocytes were located mostly in zone (b), where the earliest evidence of Ig synthesis was found.

The large macrophages in zone (a) are not dendritic and are thought to play only a secondary role in follicular antigen capture (Nossal, Abbot, Mitchell & Lummus 1968). There is no evidence that they phagocytose material from the crypt or that they migrate from the crypt to the follicle centre. The tingible bodies within them are believed to consist of material from effete cells and they are probably linked in some way with the high mitotic activity of the cells of zone (a). The increase in their numbers and regular pattern of distribution in hyperplastic follicles would be in accord with this.

The finding of relatively small numbers of cells containing IgD or IgE is in keeping with previous reports (Crabbé & Heremans, 1967; Ferrarini *et al.*, 1975). Crabbé & Heremans found that D-positive cells were sometimes more numerous than M cells and that there were more D cells in tonsils than in

other lymphoid tissues. Likewise tonsils and adenoids have been shown to possess greater numbers of IgE-containing plasma cells than other lymphoid tissues (Tada & Ishizaka, 1970). These authors pointed out that the number of IgE-forming cells in lymphoid tissues was greater than would be predicted from the concentration of IgE in the serum, and that this could result from its relatively short half-life ($1\frac{1}{2}$–2 days) or from most of the IgE formed in mucosa and tonsils being secreted and not entering the blood. IgG- and IgA-positive cells were present in all of the tonsils examined by them, outnumbering IgE cells about ten times. In contrast to this, and to our own results with tonsils from what was very largely an adult population, Østergaard (1975) found no IgA cells in 30% and no IgE cells in 60% of tonsils from children aged 4–15 years; and in 25% of the tonsils both IgA and IgE were absent. IgE may play a part along with IgA in local immune defence of the mucosa of the nasopharynx and respiratory tract, and Østergaard postulated that absence of IgE and/or IgA might predispose to tonsillitis of the type from which his patients suffered, a suggestion along the same lines as that put forward for IgA by Donovan & Soothill (1973). On the other hand, the absence of both IgA and IgE cells tended to be found in the younger patients in his series, and delayed or incomplete development of the IgA and IgE systems might be the explanation of his findings. Incidentally it should be noted that although IgE is the reagin antibody on the surface of mast cells, there was no correlation in the present study between the distribution of IgE-positive cells and the mast cells scattered throughout the tonsil.

Our failure to detect IgA (SC) is not surprising, in view of the fact that IgA is not the dominant Ig in the tonsil. Moreover, the mucous glands attached to the capsule of the tonsil do not secrete into the crypts, unlike the lingual tonsil, in which mucus from a gland opening into the base of the crypt sweeps away the debris in the crypt lumen. The IgA (SC) appears to be formed by the epithelial cells of the mucous gland, and the IgA also appears to be of local origin, as judged by the presence of IgA-positive cells in the stroma of the gland. Our failure to detect IgE in the secretion of the mucous gland, and the lack of IgE-containing cells in the stroma of the gland, confirm that in this respect IgE differs from IgA. This agrees with the results of Nakajima, Gillespie and Gleich (1975) who found IgE in the nasal washings of atopic subjects but could not detect it in the parotid fluid of these or of other (non-atopic) individuals.

With regard to technique, the PAP procedure proved very satisfactory in use, and study of the tissue sections and interpretation of the appearances were much easier than with comparable FITC-labelled preparations. Initially it was feared that non-specific reactions might be troublesome, particularly in view of the tendency for plasma cells to react non-specifically when normal (non-immune) rabbit serum was used in stage (d). However when the optimum titre for the specific antisera was used for this stage, non-specific reactions either disappeared or decreased greatly, to form a constant background against which the various specific reactions were readily detected. The poor results obtained with buffered paraformaldehyde and formalin were unexpected, particularly in view of the well-recognized qualities of paraformaldehyde as a fixative for preserving cell ultrastructure; and our attempts to 'improve' fixation by adjusting osmolarity by the addition of sucrose, as is frequently done with fixatives for electron microscopy (Caulfield, 1957), gave even poorer results. The best fixatives for immunohistochemical purposes were the simplest, viz, formol-saline and aqueous formalin. The explanation may be that the better the preservation of cell structure, and especially of the plasma membrane, the more difficult it is for reagents of large molecular weight to enter the cells to react within the cell; and fixatives which fail to preserve the cell membranes are thus more suitable for immunohistochemistry, at least at the level of light microscopy. Quenching in isopentane seems to 'open up' cells and there appear to be no problems as regards penetration of reagents in sections (cryostat or paraffin) from tissues treated in this way. Others (Kuhlmann, Avrameas & Ternynck, 1974) have shown that cell membranes may act as barriers in immunohistochemical techniques and that disruption of them may be advantageous. These authors used frozen sections to achieve consistent results in their ultrastructural studies, and they pointed out that because of cross-linkages some fixatives, e.g. glutaraldehyde, may make it more difficult for labelled antibodies to gain access to antigenic determinants. In contrast to our inconsistent results with glutaraldehyde-containing fixatives, the results with freeze-drying and fixation in DEPC vapour, as advocated by Pearse *et al.* (1974) for preservation of the antigenic properties of polypeptide hormones, were impressive, the Ig-containing cells reacting to higher titres and more consistently than in tissues prepared in

other ways. Unfortunately the tissues show poor preservation of cell structure in the electron microscope (unpublished observations).

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