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Expression of nerve growth factor receptor immunoreactivity on follicular dendritic cells from human mucosa associated lymphoid tissues

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SUMMARY

Nerve growth factor (NGF) was originally considered as a trophic factor for peripheral sympathetic and sensory neurones; however, recent reports indicate that NGF may induce proliferation of immune and haematopoietic cells. Histochemical studies conducted in human spleen and lymph nodes have suggested the presence of NGF receptor (NGF-R) immunoreactive elements in secondary follicles; however the nature of the cells bearing the NGF-R in lymphoid tissue has not been determined. In this paper we report the results of an immunohistochemical study conducted on mucosa associated lymphoid tissue. Using a specific monoclonal antibody to human NGF-R (mAb 20.4) we observed an NGF-R-immunoreactive population in all secondary lymphoid follicles examined. Double immunostaining revealed that this population was composed of follicular dendritic cells (FDC); lymphoid cells within the germinal centres did not appear to be 20·4 immunoreactive. Cell suspensions from tonsillar follicles also contained NGF-R immunopositive dendritic cells which were enriched by a 20.4 labelled magnetic bead procedure, revealing cells with the morphological characteristics of FDC. Mononuclear cells from human peripheral blood did not contain any NGF-R-immunoreactive elements using our techniques.

INTRODUCTION

It has recently been shown that 2.5S nerve growth factor (NGF) acts not only on the peripheral and central nervous systems, but also on several cells belonging to the haematopoietic and immune systems. The administration of NGF to neonatal rats is known to elicit mast-cell hyperplasia.[1] A mast-cell degranulation product feedback mechanism may, at least in part, be responsible for this phenomenon, as suggested by recent *in vivo* studies.[2] According to Thorpe and Perez-Polo, NGF can enhance the mitogen-stimulated proliferation of rat spleen mononuclear cell cultures.[3] However, the cell type mediating the proliferative response and binding NGF, was not determined.

In the human, NGF has been shown by us to produce a significant stimulation of the number of colonies grown from peripheral blood mononuclear cells (PBMC) in methyl cellulose culture, which appeared to be dependent on the presence of T cells. In addition, it has been shown that NGF can induce granulocyte chemotaxis *in vitro*[4] and *in vivo*[5] and induce the expression of interleukin-2 (IL-2) receptors on cultured human PBMC.[6] The metabolic effects of NGF on several target cells are mediated by a specific cell-surface glycoprotein receptor (NGF-R).[7–9] Recently Otten *et al.*[10] have reported immunoprecipitation of NGF-R at low levels from purified B and T human lymphocytes isolated from peripheral blood and that NGF can induce a dose-dependent increase in the [^{3}H]thymidine uptake of both B- and T-cell cultures. The same authors demonstrated that NGF receptors can be immunoprecipitated from cell membrane-enriched fractions from the whole-cell population of spleen and lymph nodes. These studies strongly indicate that NGF may act as an immunomodulator directly through its specific receptor.

Other NGF receptor studies have been conducted on human lymphoid tissues. Garin Chesa *et al.*[11] demonstrated a strong NGF-R immunoreactivity in follicles of lymph nodes and spleen; this observation has been confirmed by Thompson *et al.*[12] However, the specific NGF-R positive cell type was not identified in these studies. We were interested to see if the NGF receptor was present on cells in the mucosal immune system, which differs in several ways from peripheral lymphoid tissues. The elements of mucosa-associated lymphoid tissue (MALT)

Abbreviations: AA, acetic acid ethanol; BSA, bovine serum albumin; FCS, foetal calf serum; FDC, follicular dendritic cells; HS, human serum; mAb, monoclonal antibody; MALT, mucosa associated lymphoid tissue; NGF, nerve growth factor; NGF-R, nerve growth factor receptor; NBF, neutral-buffered formalin; NSS, normal swine serum; PLP, periodate lysine paraformåldehyde; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; TBS, Tris (hydroxymethylaminomethane)-buffered saline.

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are found both diffusely and organized in structures similar to secondary follicles of other lymphatic organs; the latter are particularly abundant in tonsils and appendix and found throughout the gastrointestinal tract. These nodules contain small B cells in a corona surrounding a central germinal zone of large B lymphocytes, some T lymphocytes, macrophages and follicular dendritic cells (FDC).[13]

We describe in this report a histochemical study of human intestinal and tonsillar tissues. NGF-R positive cells were found in all secondary lymphoid follicles in the tissues examined. Further studies demonstrated that, in these sites, FDC are the predominant NGF-R positive cell type.

MATERIALS AND METHODS

Antibodies

A mouse anti-human nerve growth factor receptor antibody 20.4 (IgG1, clone 200.3.G6.4), as well as the monoclonal antibody (mAb) 1B7.11 (IgG1 anti-2,4,6,trinitrophenyl, clone T1B191) which was used as a control in the immunobead cell separation experiments, were purified from cell culture supernatants of hybridoma cell lines (American Type Culture Collection, ATTC, Rockville, MD). MAb were purified using a column of Sepharose CL-4B coupled to protein A (Pharmacia, Uppsala, Sweden). Bound Ig was eluted with glycine/HCl buffer (pH 2·5) followed by extensive dialysis against 0·5 M NaCl 0·1 M $NaHCO_3$, pH 8·3. The protein concentration was assessed by optical density. A mAb reactive with human follicular dendritic cells,[14] designated R4/23 (IgM isotype), was obtained from Dako (Dimension Laboratories, Mississauga, Ontario, Canada). MAb Leu-M1 (IgM isotype, cluster antigen CD15) was purchased from Becton Dickinson (Mississauga, Ontario, Canada) and used as control in the double immunostaining.

NGF-positive cell line

A human NGF-R positive malignant melanoma cell line, SK. MEL.28, was purchased from ATCC (cat. no. HTB 72) grown in 10% foetal calf serum (FCS) containing RPMI-1640 medium (10% FCS RPMI) harvested, washed three times in RPMI and resuspended in 10% human serum in 0·05 M Tris-buffered saline solution, pH 7·6 (10% HS/TBS). Cytospin smears were prepared, fixed in 10% neutral-buffered formalin (NBF) for 15 min at 4° and used as a positive control in immunocytochemical studies.

Specimens

Palatine tonsils ($n=20$), from children aged 2–14 years, were obtained immediately after surgical resection and promptly fixed in either 10% NBF for 24 hr or acetic acid-ethanol (1:9, AA) for 5–6 hr, before routine processing to paraffin. Some pieces of tonsils were frozen for cryostat sectioning or used for cell isolation.

Vermiform appendices ($n=5$) removed during major abdominal surgery, and uninvolved areas of resected intestines ($n=10$) from a variety of gastrointestinal disorders, were also studied. The specimens included colon, ileum and jejunum. For immunohistochemistry, only sections containing lymphoid aggregates in a haematoxylin/eosin stained section slides were employed.

Immunocytochemistry

Single staining. Tissue sections were deparaffinized and endogenous peroxidase activity was inactivated by incubation in 0·5% hydrogen peroxide in methanol for 30 min. Neutral-buffered formalin (NBF) fixed slides were then digested in 0·1% trypsin (type II crude, Sigma Chemical Co., St Louis, MO) for 10 min at 37°. Acetic acid ethanol (AA) fixed tissues were not pretreated. Non-specific staining was blocked by preincubation with non-immune rabbit serum (10% NRS in TBS) for 30 min, the primary antibody was applied (20.4 at 1·14 μg/ml or R4/23 at 5·7 μg/ml) and sections were incubated for 1 hr. In control slides the primary antibody was omitted. Reagents obtained from the Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA) were then applied. Biotinylated rabbit anti-mouse Ig and streptavidin-peroxidase conjugate were applied sequentially, each slide receiving a double cycle of reagent applications. The slides were then developed in 0·02% aminoethylcarbazole in 0·02% hydrogen peroxide in 0·05 M acetate buffer pH 5·0 (AEC solution: Sigma, cat. no. A-5754). Red staining was visible after 15 min and slides were then counterstained in haematoxylin and mounted using an aqueous mounting medium. All the incubations were performed at room temperature.

Double immunostaining. Combined 20.4 and R4/23 immunoreactivity was demonstrated by sequential application of the mAb in AA fixed gut and tonsil sections. After dewaxing, endogenous peroxidase activity was inactivated by incubation in 0·5% hydrogen peroxide in methanol for 30 min and non-specific staining was blocked by application of 20% normal swine serum in TBS (20% NSS/TBS) for 30 min. This was followed by mAb 20.4 (1·14 μg/ml in 10% NSS/TBS) for 30 min. In control slides this was substituted by diluent only. Two cycles of rabbit anti-mouse Ig (1/100 in 10% NSS/TBS; Dako) and peroxidase conjugated swine anti-rabbit Ig (1:100 in 10% NSS; Dako) were then applied. The slides were developed in AEC solution until a sharp red staining was obtained (15 min). The second part of the double immunostaining was performed to localize FDC. In order to decrease non-specific binding of avidin-biotin to the tissue, slides were treated with reagents from the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Incubation with avidin solution (15 min) was followed by biotin solution (15 min). Slides were then rinsed and 20% NSS/TBS was applied (20 min). Test slides received mAb R4/23 (5·7 μg/ml in 5% HS/TBS), the control slides received either the mAb Leu-M1 (IgM, at the same concentration in 5% HS/TBS) or diluent alone. Two cycles of a biotinylated rabbit anti-mouse IgM (Zymed, 1:500 in 5% HS for 10 min) followed by alkaline phosphatase conjugated streptavidin (Zymed 1:50 in TBS for 5 min) were then employed. Slides were developed using Fast Blue BB salt (Sigma, cat. no. F-3378, 1 mg/ml final concentration) in Naphthol AS-BI solution. Levamisol hydrochloride (Sigma, cat. no. L-9756; 0·25 mg/ml final concentration) was added to block endogenous alkaline phosphatase activity. All the procedures were performed at room temperature.

Dispersion of tonsillar follicle cells

Cell suspensions from dissected tonsillar follicles were prepared according to a method previously reported by Lilet-Leclercq *et*

al.[15] and modified by Tsunoda & Kojima.[16] Briefly, follicles were isolated from thinly sliced tonsil under a dissecting microscope and submitted to four sequential enzyme treatments: one application of 0·05% collagenase type I (Sigma) in RPMI; and three cycles of a mixture of 0·05% collagenase (type I Sigma), 0·04% deoxyribonuclease (Sigma), 0·05% dispase (type I Boehringer Mannheim, Mannheim, Germany) in phosphate-buffered saline (PBS) for 15 min at 37°. After each treatment the free cells were collected and stored in 5% FCS/PBS at 4°. Aliquots of the whole-cell suspension were used to prepare cytospin smears.

To concentrate FDC, free cells, resuspended in PBS, were layered on a 30% FCS/PBS column and, after 60 min at unit gravity, the upper fraction (FDC depleted) and the lower fraction (FDC enriched) were collected. Cytospin smears were then prepared and fixed in 10% NBF for 15 min at 4°.

Some cell pellets were fixed in 2% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, for 2 hr, followed by 1% osmium tetroxide for 1 hr. The pellets were embedded in Spurr's resin and the sections were stained with uranyl acetate and lead citrate and observed in a Jeol 1200EX transmission electron microscope at 80 kV.

Positive selection of mAb 20.4 reactive cells

In order to isolate specifically NGF-R positive cells, the mixed cell population was preincubated with mAb 20.4 (1·14 μg/ml) or 1B7.11 (at the same concentration) as negative control, in 0·1% bovine serum albumin (BSA) PBS (0·1% BSA/PBS) for 30 min at 4°. The cells were then washed three times in RPMI, pH 7·4. The pellet was resuspended in 0·1% BSA/PBS and mixed gently for 60 min with 3×10^6 magnetic beads coated with goat anti-mouse IgG (Dynabeads TM M-450, Dynac Inc., New York, NY) at 4° by tilt rotation. Cells rosetted with magnetic beads were harvested by a magnetic field (Dynal Magnet: MPC 6), resuspended in 1 ml of PBS, examined as cytosmears and counted in a haemocytometer.

RESULTS

Distribution of 20.4 immunoreactivity in MALT

Immunohistochemistry on tonsil and gut sections showed that mAb 20.4 stained lymphoid follicles. The staining was limited to the germinal centres and presented a reticular pattern highly suggestive of a distribution on FDC (Fig. 1). This distribution was observed in cryostat sections as well as in both 10% NBF and AA fixed paraffin embedded tissues. Other 20.4 positive elements were observed: in the gut sections there were strongly immunoreactive structures that showed morphological characteristics of neural elements (Fig. 1). In the tonsil the basal epithelial layer was positive but neural elements were not detected (Fig. 2). When R4/23 immunoreactivity was demonstrated on the same tissues, the dendritic processes of FDC were revealed as a reticular network inside the germinal centres; cellular bodies of FDC were not easy to identify. Neither the tonsillar epithelium nor the small nerve fibres were stained with the mAb R4/23.

FDC are the predominant 20.4 reactive cell population in lymphoid follicles

When thin serial sections of gut and tonsil were stained consecutively using 20.4 and R4/23 antibodies, a very similar

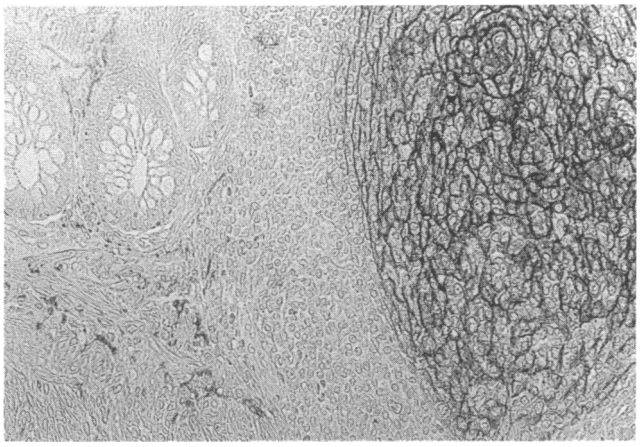

Figure 1. Immunohistochemical demonstration of mAb 20.4 reactivity in AA fixed human colon, showing prominent staining of a lymphoid follicle. In the interfollicular lamina propria the pattern of the staining corresponds to the distribution of nerve fibres (original magnification ×250).

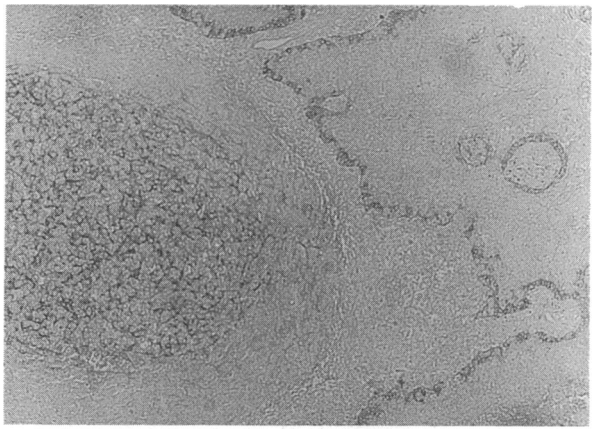

Figure 2. Immunochemical demonstration of mAb 20.4 reactivity in AA fixed human tonsil. All follicles exhibited strong staining localized to the germinal centres. Immunoreactivity is also present in the basal layer of the epithelium (original magnification ×100).

pattern of staining was observed in germinal centres; however the staining patterns did not completely overlap, suggesting some differences in the distribution of antigens on the germinal centre cells. The double immunohistochemical technique showed that the majority of FDC were stained positive with 20.4 and R4/23 (20.4^+, $R4/23^+$), being visualized by a purple staining (Fig. 3). Small subpopulations of 20.4^+, $R4/23^-$ (red staining) and 20.4^-, $R4/23^+$ (blue staining) FDC were also identified and appeared to be localized to the periphery of germinal centres (Fig. 4). To demonstrate that cross-reactions were not occurring, control slides were used in which mAb R4/23 was substituted by mAb Leu-M1 (anti-human myelomonocytic antigen, CD15) at the same concentration or by diluent (Fig. 3c). In these slides, the 20.4 immunoreactivity was not contaminated by blue staining due to non-specific binding of reagents to the tissue, showing that the colour obtained in test

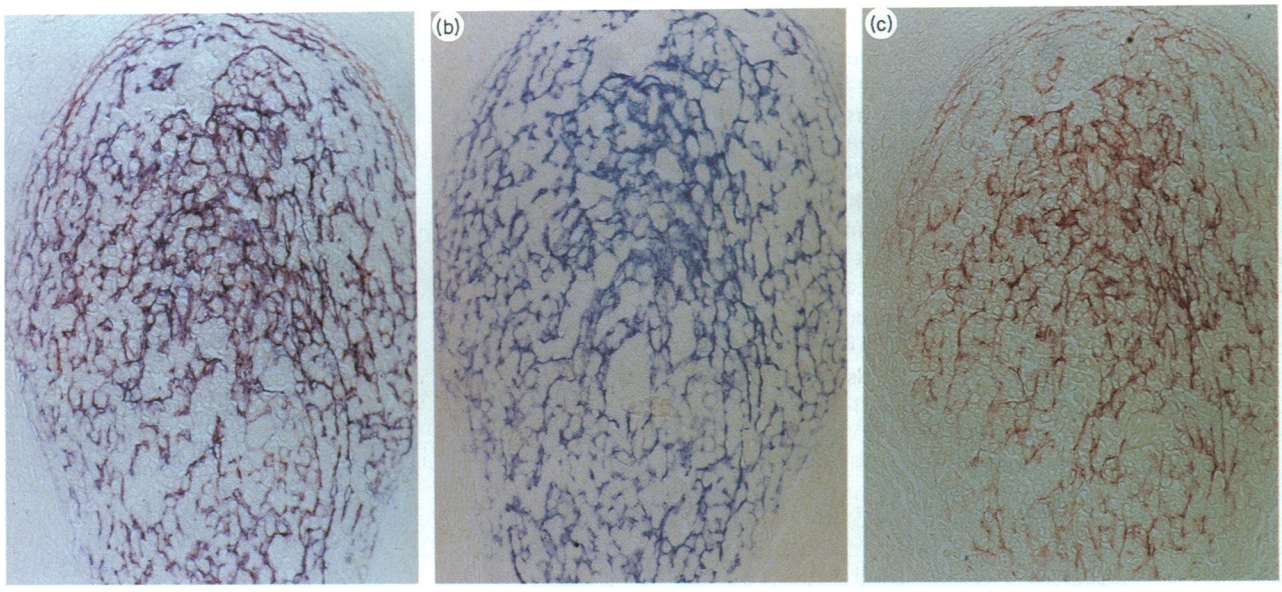

Figure 3. Immunohistochemical localization of mAb 20.4 and mAb R4/23 in AA fixed colon follicles (original magnification × 500). (a) MAb 20.4 (red) and R4/23 (blue) were used sequentially in a biotin-streptavidin method. FDC appear to bind both antibodies. The cells trapped by the cytoplasmic processes of FDC do not appear reactive with either mAb. (b) When mAb 20.4 was omitted from the sequence of the double-immunostaining technique, an R4/23 positive (FDC) blue reticular pattern was observed. (c) The substitution of the mAb R4/23 by the class-matched mAb Leu-M1 in the double-staining procedure did not produce any blue staining in the lymphoid follicles. Scattered blue granulocytes were noticed in adjacent areas of the mucosa.

slides originated from a combination of binding of 20.4 and R4/23. Slides treated with mAb Leu-M1 presented, as expected, scattered blue stained leucocytes (not shown).

Characterization of dispersed tonsillar follicle cells

The enzymatic digestion of the tonsil follicles produced a cell suspension constituted by small clumps and isolated cells.

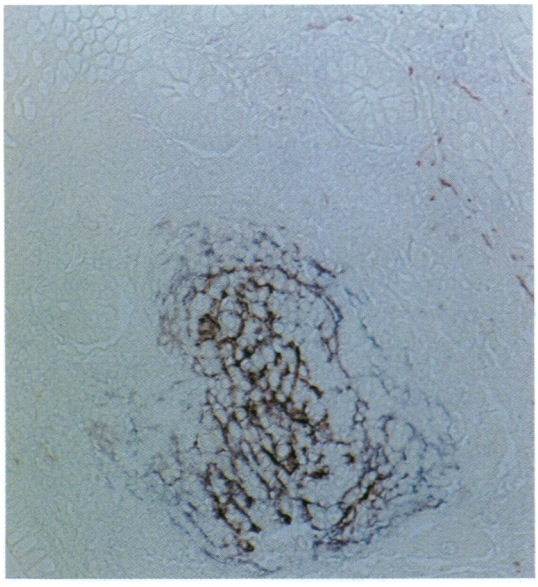

Figure 4. Lymphoid follicle in human colon (AA fixed). Immunostaining for mAb 20.4 and mAb R4/23. The majority of FDC appear to be stained by both mAb; however, at the periphery of the follicle some blue (mAb 20.4 negative-mAb R4/23 positive) FDC are visible (original magnification × 250).

Cytospin smears, processed for immunocytochemistry using mAb 20.4, showed an immunoreactive subpopulation composed of free and clumped cells. The most evident feature of the isolated immunoreactive cells was their large size, the presence of one or two nuclei and the cytoplasm, which usually had long processes, conferring a dendritic appearance. Inside the clumps, the immunoreactive cells appeared large but the cytoplasmic processes were more difficult to identify because they were partially obscured by other cells included in the cluster. Mononuclear peripheral blood cytospin smears were tested in the same experimental conditions with consistently negative results, whereas the human melanoma cell line SK.MEL.28 presented strong immunoreactivity.

The presence and the integrity of FDC in dispersed tonsillar follicle cells was confirmed by electron microscopy (Fig. 5),

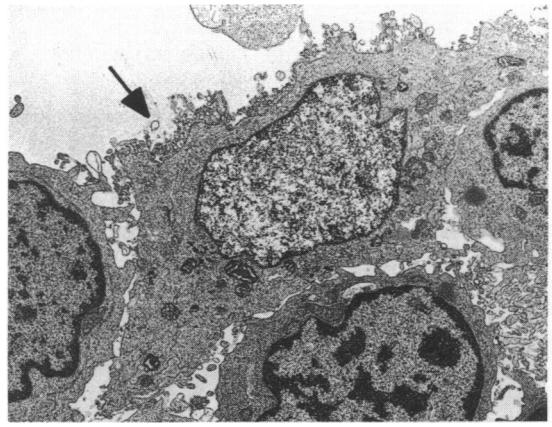

Figure 5. Electron micrograph of tonsillar follicular dendritic cell suspension. A typical FDC (arrow) with cytoplasm extending between small lymphoid cells is shown (original magnification × 7000).

which showed the presence of large cells having one or two clear, indented nuclei with dispersed chromatin. A small ring of cytoplasm containing several mitochondria was visible around the nuclei. Delicate cytoplasmatic extensions were easily identified in both single FDC and those within aggregates.

A sedimentation step performed on dispersed tonsil cells according to the methods of Tsunoda and Kojima[16] resulted in upper and lower fractions. When tested for R4/23 and 20.4 immunoreactivity, the upper fraction was negative for both mAb, whereas the lower fraction contained FDC R4/23 as well as 20.4 positive cells. Both the R/4/23 and the 20.4 immunoreactive cells were occasionally found isolated and more frequently in clumps, enveloping smaller cells with fine cytoplasmatic processes.

Positive selection of 20.4 reactive cells

The use of mAb 20.4 and anti-mouse IgG coated magnetic beads allowed us to select NGF-R positive cells from the whole follicular population. The proportion of individual 20.4 positive cells recovered using this technique represented a minority of the original follicular population, ranging from 0·2 to 0·9% in four different experiments. Isolated cells associated with beads had a clear cytoplasm; many had apparent cytoplasmic processes. One or two nuclei were often detectable.

DISCUSSION

In this study we document the presence of prominent NGF-R immunoreactivity in human MALT, localized mainly on germinal centre FDC. These observations were made on gut and tonsil tissue sections and on dispersed tonsillar follicular cells, using a monoclonal antibody to the human NGF receptor (20.4) and mAb R4/23, recognizing FDC.

Several earlier studies assessed NGF-R immunoreactivity in normal and neoplastic human tissues. They also employed immunohistological techniques and NGF receptor mAb. Immunoreactivity was detected in a proportion of neuroectoderm derived-tumours and carcinomas[11] whereas non-neurogenic mesenchymal tumours were generally negative.[17] Among normal tissues, NGF-R immunoreactivity has previously been localized to neuronal axons, Schwann cells and perineural cells of both sensory and sympathetic peripheral nerves. Several epithelia are also known to be immunoreactive, including the basal epithelium of oral mucosa, tongue and bronchioles. Myoepithelial cells in salivary gland, eccrine sweat glands and mammary gland are also NGF-R immunoreactive. According to these studies all mucosal surfaces seem to contain a high number of NGF-R bearing structures.

With regard to lymphoid tissues, the NGF-R immunoreactivity of follicular germinal centres in human spleen and lymph nodes was first reported by Garin Chesa *et al.*[11] and more recently confirmed by Thompson *et al.*,[12] however, neither group investigated which cell type(s) may be expressing the receptor.

Our immunohistochemical studies employed mAb 20.4 to localize NGF-R and confirm these studies; and, by the use of R4/23 as a marker for FDC, to extend them to identify the specific NGF-R bearing cell type. We considered R4/23 a suitable marker for FDC in our study because of its ability to react strongly with FDC in the entire area of human germinal centres. Furthermore, it has been shown that B cells located in the FDC network are unreactive with R4/23[14] and other structures within the tonsillar tissues have not been shown to bind R4/23.[14]

The NGF-R immunoreactivity appeared as a complex reticulum between germinal centre lymphoid cells. Thin (1 μm) sections permitted a partial resolution of the cellular profiles, suggesting that FDC expressed NGF-R immunoreactivity. Serial sections stained alternately for 20.4 and R4/23, showed an almost coincident pattern of germinal centre immunoreactivity, especially in follicle centres. In both gut and tonsil specimens, subpopulations of FDC marked by only one of the two mAb were found at the periphery of follicular germinal centres. The use of the double-immunostaining technique demonstrated that the majority of germinal centre cells identified by 20.4 were also reactive with R4/23. An internal control was provided by the presence, in each slide, of elements reacting only with 20.4: the basal layer of the epithelium in tonsil, and nerves in gut lamina propria. These structures maintained their original red staining after slides had undergone the complete double-staining procedure.

When whole-cell suspensions from tonsil follicles were prepared and cytosmears immunostained using either mAb R4/23 or mAb 20.4, the two subpopulations detected appeared to have similar dimensions, cytoplasmic processes and one or two nuclei. The use of immunocoated magnetic beads allowed us to recover a subpopulation of NGF-R positive cells representing 0·2–0·9% of the total cell suspension.

It is known from animal studies, using a sensitive immunoassay for NGF[18] that NGF is produced by a variety of tissues, including salivary glands, and as such may be locally available to tonsil tissue. NGF is also found in all sympathetically innervated tissues, with the concentration of NGF detected being proportional to the density of innervation.[12] In this regard it is reasonable to consider that NGF may be locally available to immune and inflammatory cells during normal or pathological situations. It is known that alterations in immune status can affect the production of NGF. For instance macrophages can elicit, through the release of IL-1, the synthesis of NGF mRNA in sciatic nerve preparations following Wallerian degeneration *in vivo*.[19] We have no information at present concerning the possible effects of altered immune function during inflammation on NGF-R expression.

FDC are known to present antigens in the form of surface immunocomplex coated bodies, known as iccosomes.[20] These are subsequently released inside the germinal centre and are available to B cells for processing and presentation to T lymphocytes. Through this mechanism, FDC co-operate in antibody production and memory-cell formation.[21] The possibility that NGF may interact with NGF-R on FDC and alter their functional properties is intriguing, particularly when considering that FDC are known to enhance the *in vitro* survival of lymphoid cells as well as to increase the proliferation activity of mitogen-stimulated lymphoid cells.[21] In view of these reports and our data, we hypothesize that the NGF proliferative effect on unfractionated rodent spleen cells, shown by Perez-Polo *et al.*, may be mediated by FDC.[6]

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