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Isolation of follicular dendritic cells from human tonsils and adenoids

III. ANALYSIS OF THEIR F_C RECEPTORS

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Accepted for publication 9 November 1984

Summary. Follicular dendritic cells (FDC), isolated from human tonsils or adenoids, were tested for their capacity to retain monomeric, aggregated or antigen-bound human antibodies in the absence of serum.

FDC retain fluorescein-labelled heat-aggregated human immunoglobulins, but not monomeric ones nor fluorescein-labelled $F(ab')_2$ in monomeric or aggregated form. Ultrastructural observations showed that colloidal gold-labelled monomeric, or antigen-bound, antibodies directed against tetanus toxoid are retained by dendrites and membrane infoldings of FDC but are never located in cytoplasmic vesicles. This retention was inhibited by incubating FDC with unlabelled aggregated or antigen-bound antibodies.

When gold-labelled anti-tetanus toxoid antibodies were incubated in the presence of protein-A before the contact with FDC, a strong reduction of their retention occurred. This further suggested the presence of Fc receptors on isolated tonsillar FDC. Endocytosis was not observed in isolated FDC, even after prolonged incubation in presence of labelled immune complexes: their Fc receptors are, thus, not related to a phagocytic activity as they are in macrophages.

Simultaneous ultrastructural labelling of Fc and C3b receptors with colloidal gold particles of different sizes did not reveal any clear relations between these two receptors on the surface of FDC.

Abbreviations: Anti-BSA-gold, colloidal gold-labelled mouse antibodies against bovine serum albumin; Anti-T-T-gold, colloidal gold-labelled human antibodies against tetanus toxoid; Anti-T-T-gold-T-T, immune complexes formed of anti-T-T-gold and tetanus toxoid; FDC, follicular dendritic cells; Ig, immunoglobulins.

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INTRODUCTION

During humoral immune reactions, contacts between lymphoid cells occur either to enhance or to depress the response. During activation, the intervention of accessory cells, such as macrophages, is accepted but remains unprecise during the following steps (proliferation and differentiation). For thymus-dependent antigens, the germinal centres appear to be the central site of proliferation and, in part, for differentiation of B cells. Inside the germinal centres the follicular dendritic cells (FDC) constitute a particular framework, but their nature and origin are ill-defined. FDC retain antigens (White, 1963; Nossal *et al.*, 1965) in the form of immune complexes (Herd & Ada, 1969; Radoux *et al.*, 1984b). This retention occurs via the C3b receptor (Papamichail *et al.*, 1975; Romball, Ulevitch & Weigle, 1980; Gerdes & Stein, 1982) or the Fc receptor (Herd & Ada, 1969; White *et al.*, 1975).

Our group succeeded in isolating FDC from

children's tonsils and adenoids. They appeared as clusters enveloping lymphoid cells (Lilet-Leclercq *et al.*, 1984; Heinen *et al.*, 1984). These FDC reacted with specific antibodies directed against human FDC or with antibodies against monocytes/macrophages; in addition, they bear HLA-DR antigens (Heinen *et al.*, 1984). Isolated FDC also bore various Ig isotypes except IgD on their surface. Using the same anti-C3b antibodies as Gerdes & Stein (1982), we showed, at ultrastructural level, that isolated FDC possess numerous C3b receptors on dendrites and inside membrane folds (Heinen *et al.*, 1984).

Fc and C3b receptors generally permit the capture and internalization of Ig by macrophages and lymphocytes (Davies & Metzger, 1983; Fearon & Wong, 1983). However, FDC are unable to endocytose their retained Ig; they therefore play different roles as accessory cells than macrophages in the germinal centres. In order to analyse this, we intend to verify the presence of Fc receptors, and to investigate their distribution and density on the surface of FDC using free or aggregated Ig or immune complexes. In the present study, we used purified human polyclonal anti-tetanus toxoid antibodies, linked these to colloidal gold particles, and tested, in various conditions, Fc receptors of isolated tonsillar FDC. Controls were performed with the aid of fluorescein-labelled human antibodies or with protein A which blocks the Fc parts of Ig.

MATERIAL AND METHODS

Isolation procedure of FDC

We have previously described the isolation procedure of FDC (Lilet-Leclercq *et al.*, 1984). In short, lymphoid follicles dissected from children's tonsils or adenoids were treated with collagenase, dispase and deoxyribonuclease. The cells were then separated by sedimentation at 1 *g* in a MEM solution (Dulbecco) containing 1·5% bovine serum albumin. Sedimented cells were centrifuged and used for the present experiments. They contained about 50,000 FDC organized in clusters mixed with free cells (macrophages and lymphocytes) or epithelial fragments in proportions varying from 1 in 200 to 1 in 1000.

Demonstration of Fc receptors

The presence of Fc receptors was tested using human anti-tetanus toxoid antibodies labelled with colloidal gold particles (anti-T-T-gold) or fluorescein-labelled human Ig or $F(ab')_2$ fragments.

Human anti-T-T were purified by affinity chromatography with a CNBr-activated Sepharose 4B column (Pharmacia Belga). Elutions were performed with NH_4CNS (3 M, pH 5) then with urea (6 M, pH 3). The solution was dialysed, concentrated and lyophilized. Purity was tested by polyacrylamide gel electrophoresis in SDS, as well as by immunoelectrophoresis.

Colloidal gold labelling was done according to the procedure described by Horisberger (1979), as modified by De Mey (1983), using PEG instead of BSA as a secondary stabilizer.

The antibodies, dialysed against 2 mM borax, pH 9, and centrifuged at 100,000 *g* for 1 hr at 4°, were added to the 20 nm gold particles (pH 9). PEG 1% was added as a secondary stabilizer and three alternate centrifugations were used to wash and concentrate the probes. Before use, the labelled antibodies were centrifuged at 250 *g* for 20 min in order to eliminate aggregates.

Immune complexes were formed by incubating 40 μl of this anti-T-T-gold solution (about 5 μg Ig) with 7·5 μg tetanus toxoid for 1 hr at 37°. Smears of these preparations on collodion-covered grids were examined by electron microscopy (Radoux *et al.*, 1984b).

Fluorescein-conjugated human IgG and fluorescein-conjugated human $F(ab')_2$ fragments were provided by Cappel Laboratories (Malvern, U.S.A.) and used at 5 and 2 mg/ml, respectively, after centrifugation at 100,000 *g* for 30 min.

Aggregation of Ig was produced by incubation at 63° for 30 min. The suspension obtained was then centrifuged at 5000 *g* for 30 min to eliminate large aggregates.

Cells were incubated in the presence of labelled Ig, free, aggregated or in the form of immune complexes at 37° for 30 min in absence of complement factors (MEM solution containing 0·4% bovine serum albumin). The cells were then centrifuged and rinsed twice in a physiological solution before examination with an optical (Leitz, MPV) microscope or an electron microscope (Philips 301).

Controls were performed by incubating cells at 37° for 30 min with unlabelled human immune complexes (13 mg/ml) before contact with the labelled immune complexes. Other controls were incubated with free or aggregated human Ig (13 mg/ml; 30 min; 37°; Croix Rouge de Belgique) before contact with the labelled Ig.

Retention of goat anti-mouse or mouse anti-BSA antibodies linked to gold particles, free PEG 2000 stabilized colloidal gold (20 nm in diameter) or

colloidal carbon (indian ink; Pelikan) was tested in similar experimental conditions. Free colloidal gold also was used with 5% horse serum which neutralizes its surface charges.

Protein A-anti-T-T-gold complexes

The fixation of anti-T-T-gold by Fc receptors was further investigated using protein A linked to anti-T-T-gold. Protein A (10 μg; Sigma, St Louis, MO) was incubated in presence of 10 μg anti-T-T-gold (37°; 30 min). In one experimental condition, it was followed by an incubation with 13 mg/ml human Ig (37°; 30 min) in order to mask all free binding sites of protein A.

Incubation of these solutions with isolated cells was performed at 37° for 30 min in a MEM solution containing 0·4% bovine serum albumin and 0·1% NaN_3. The cells were then centrifuged, rinsed twice, fixed and observed with an electron microscope.

Controls were performed by incubating cells with anti-T-T-gold alone in the same conditions.

Simultaneous labelling of Fc and C3b receptors

Isolated FDC were first incubated (30 min; 37°) with anti-T-T-gold, rinsed and then immunolabelled with an anti-C3b receptor monoclonal antibody (TO5) according to a technique described previously (Heinen *et al.*, 1984), using goat anti-mouse Ig gold-labelled (5 nm in diameter) antibodies (GAM-G5; Janssen, Life Sciences Product, Beerse, Belgium).

Electron microscopy

Isolated cells were fixed in 2·5% glutaraldehyde (in cacodylate buffer; 0·1 M; pH 7·2), postfixed in OsO_4 (2%) and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate and lead citrate. Observations were made with a Philips 301 microscope at 80 kV.

RESULTS

Analysis of the Fc receptors by optical microscopy

Living FDC isolated from tonsils or adenoids were incubated in a physiological solution containing human aggregated IgG labelled with fluorescein for 30 min at 37°. Fluorescence labelling (Table 1) was found on FDC, as well as on some free lymphocytes and macrophages. When FDC were preincubated with unlabelled human aggregated Ig, strong reduction of fluorescence labelling was produced (Table 1).

Monomeric human fluorescein-labelled IgG or $F(ab')_2$ fragments were not retained on FDC, at least as detectable by fluorescence microscopy (Table 1).

Analysis of the Fc receptors at ultrastructural level

Living FDC isolated from tonsils or adenoids were placed in the presence of human anti-T-T-gold free or in the form of immune complexes for 30 min at 37°. The results are summarized in Table 2. Free or antigen-conjugated anti-T-T-gold were retained in a nearly similar manner on FDC, but also on some lymphocytes and macrophages which showed endocytosis of gold particles contrary to FDC. In comparison to lymphocytes and macrophages, FDC fixed a larger number of anti-T-T-gold particles along microvilli or inside tubular or lamellar membranous folds (Figs 1 and 2). Preincubation with human immune complexes diminished the gold labelling of FDC more strongly than human aggregated Ig (Table 2).

When FDC were incubated in the presence of anti-T-T-gold for 30 min at 37°, and then rinsed and placed for an additional 1 hr in an anti-T-T-gold-free medium, no endocytosis could be observed but some loss of gold particles occurred.

Heterologous, goat anti-mouse antibodies labelled

Table 1. Retention of fluorescein-labelled IgG or $F(ab)'_2$ fragments by isolated FDC

	Without preincubation	After preincubation with:	
		Free Ig	Aggregated Ig
Free IgG-FITC	−	−	−
Aggregated IgG-FITC	++a*	+	−
Free $F(ab)'_2$-FITC	−	−	−
Aggregated $F(ab)'_2$-FITC	−	−	−

*(++) or (+) represent high and low fluorescence positivity, respectively.

Table 2. Retention of free or antigen-conjugated human anti-T-T-gold, mouse anti-BSA-gold, free colloidal gold or indian ink by isolated FDC

	Without preincubation	After preincubation with:	
		Aggregated Ig	Immune complexes
Anti-T-T-gold	++ to +++*	+ to ±	± to −
Anti-T-T-gold-T-T	+++	+ to ±	± to −
Mouse anti-BSA-gold	−	NT†	NT
Free colloidal gold	+	NT	NT
Free colloidal gold + serum	−	NT	NT
Colloidal carbon	+	NT	NT

* (±) to (+++) represent estimations of the number of gold particles retained per FDC section: (±) 1 to 10; (+) 10 to 50; (++) 50 to 100; (+++) more than 100 particles.

† NT, not tested.

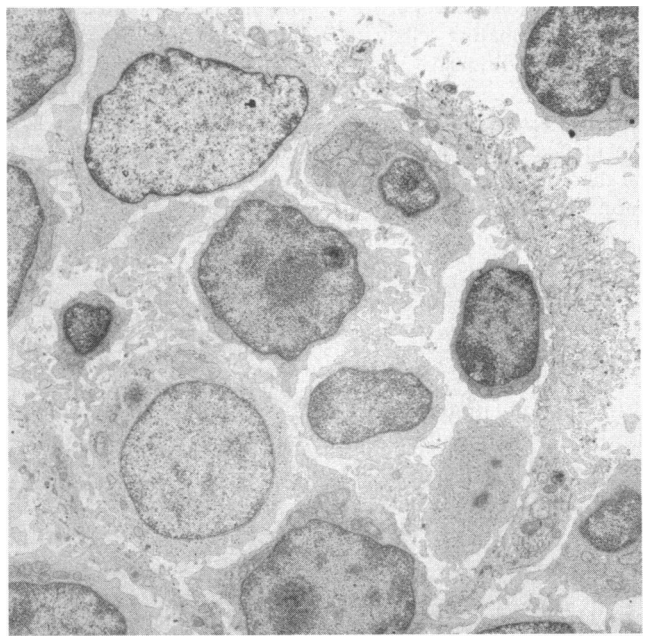

Figure 1. Ultrastructural aspect of an isolated FDC. The nucleus can be seen at the upper left side. It surrounds lymph cells by cytoplasmic extensions retaining gold-labelled homologous antibodies (see also Fig. 2). (Magnification × 3300.)

with colloidal gold were not retained by FDC (Fig. 3). Free colloidal gold particles (20 nm diameter) were slightly retained (as was colloidal carbon) but only when added to cells without serum proteins.

Living, isolated FDC were treated with anti-T-T-gold linked to protein A. In order to block free binding sites on protein A which would react with Ig retained on the surface of FDC, in a supplementary experiment, we added free human Ig before the contact with isolated FDC. Only this last experimental condition showed no or weak immunogold labelling (Table 3).

Relation of Fc to C3b receptors

The same living FDC were incubated first with anti-T-T-gold (20 nm) and then with antibodies

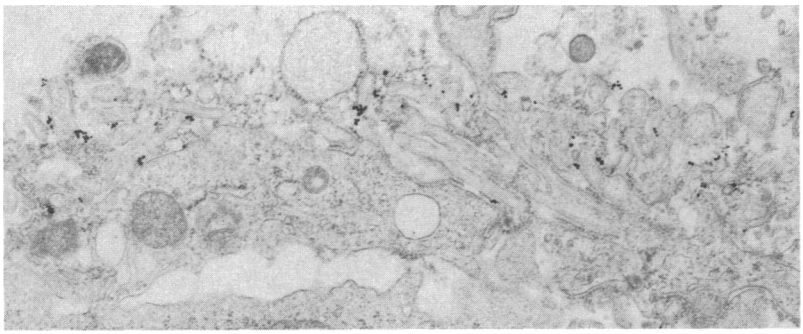

Figure 2. Enlargement of Fig. 1: FDC cytoplasmic extensions are covered with dendrites retaining gold-labelled anti-T-T immune complexes. (Magnification ×20,250.)

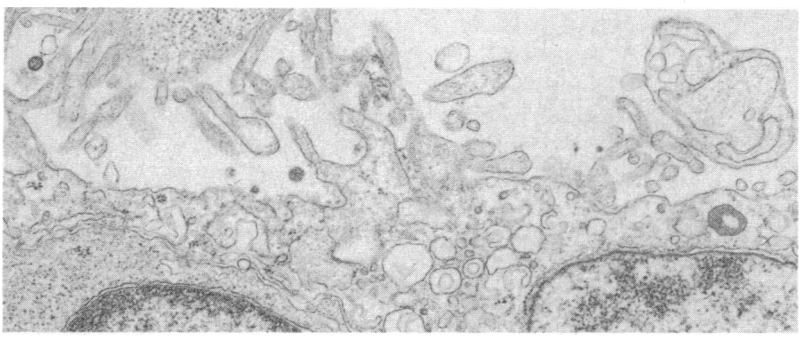

Figure 3. High magnification of a human FDC treated with heterologous goat anti-mouse gold-labelled antibodies. No retention can be seen on its cytoplasmic extensions. (Magnification ×22,500.)

Table 3. Retention by isolated FDC of anti-T-T-gold free or fixed on Protein A

	Retention on FDC
Protein A-anti-T-T-gold	++*
Protein A-anti-T-T-gold + human monomeric Ig	± to −
Anti-T-T-gold	++ to +++

*(±) to (+++) represent estimations of the number of gold particles retained per FDC section: (±) 1 to 10; (+) 10 to 50; (++) 50 to 100; (+++) more than 100 particles.

directed against C3b receptors (T05) immunolabelled with goat anti-mouse gold labelled (5 nm) antibodies before processing for electron microscopy (Fig. 4). All FDC bore both markers (20 nm and 5 nm gold particles), but there was no appearance of an association between the two markers which were found randomly distributed mainly along the periphery.

DISCUSSION

Our results demonstrate that living isolated FDC are able to retain human aggregated Ig or immune complexes via their Fc receptors. This retained material was found at the surface of their dendrites or inside membrane folds but never in cytoplasmic vacuoles.

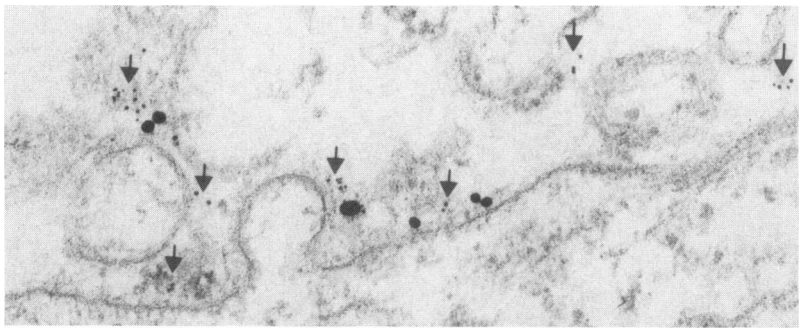

Figure 4. High magnification of the external surface of a FDC treated first with anti-T-T-gold-labelled antibodies (20 nm) and the immunolabelled for the C3b receptors with 5 nm colloidal gold particles (arrows). The two labels show independent distribution. (Magnification × 78,750.)

Conflicting results were obtained with monomeric Ig fixed on colloidal gold particles which were retained by FDC, but this was not detected with fluorescein-labelled Ig. The fixation on colloidal gold (the nature of this fixation is unclear) apparently induces configurational changes of monomeric Ig, rendering them able to fix to Fc receptors. Further studies at ultrastructural level with another marker may clarify the fixation of monomeric Ig by FDC and will also indicate the isotypes retained by the Fc receptors.

Free colloidal gold particles were retained when added without serum proteins; this may be due to surface charges of these particles reacting with membrane proteins in a way similar to colloidal carbon. When these charges were neutralized by the addition of horse serum proteins, no retention of colloidal gold particles was seen. Fixation of Ig on colloidal gold neutralizes the surface charges: this was verified by the use of gold particles covered with mouse or goat Ig which are not retained by FDC. This last point also shows the specifity of these receptors for homologous Ig.

Protein A-anti-T-T-gold complexes were fixed on FDC surfaces when the free binding sites on protein A were not blocked by free Ig. Thus, isolated FDC bear Ig on their surface, whose Fc fragments are free. Indeed, these may be retained via the C3b receptors which are known to exist on FDC (Gerdes & Stein, 1982; Heinen *et al.*, 1984).

According to our double-labelling experiments, there are no clear configurational relationships between Fc and C3b receptors; however, our labelling technique does not necessarily reveal all present receptors and only marks a short period of time of the membranous state of isolated FDC. Nevertheless, an analogous observation was made by Michl *et al.* (1983) who observed independent migration of Fc and C3b receptors in macrophages.

We have no clear indications concerning the functions of the Fc or C3b receptors found on FDC: they are not related to any endocytotic phenomenon as they are in macrophages or lymphocytes (Davies & Metzger, 1983; Fearon & Wong, 1983). Suzuki, Sadavasian & Taki (1981) reported that Fc receptors after aggregated IgG fixation exhibit phospholipase A_2 activity which may be concerned with secretion of prostaglandins; FDC may, perhaps, exert a similar secretion activity in relation to their Fc receptors.

The retained aggregated Ig or immune complexes may exert a role during the thymus-dependent antigen response in the germinal centres but, again, only hypotheses exist: Tew, Phipps & Mandel (1980) consider that they intervene for the antibody secretion regulation. Klaus *et al.* (1980) suggested that they regulate B memory cell formation. Kunkl & Klaus (1981) thought that they selectively trigger high-affinity precursor cells. Other authors have suggested that FDC only work as filters retaining various substances non-specifically (Chen *et al.*, 1978; Curran, Gregory & Jones, 1982). We think that the specific retention of Ig via the Fc or C3b receptors (which are densely present on FDC membranes) plays a part in the creation of a microenvironment necessary for activation, proliferation or differentiation of B lymphocytes, either in inducing secretion of substances, or in presenting antigens or antibodies, may be anti-idiotypic antibodies, which regulate the B memory cell or the antibody-producing cell formation. Indeed, according to Kölsch *et al.* (1983), immune complexes block the differentiation of B lymphocytes into plasma

cells, creating a positive signal for B memory cell formation. We stated previously that FDC trap immune complexes and conserve them for a period of time in deep membranous infoldings apparently not in direct contact with the surrounding cells (Radoux *et al.*, 1984a). The reason for this phenomenon is unclear. It may either protect lymphocytes from an excess of immune complexes or constitute a reserve for continuous stimulation. Indeed, C3 fragments may inhibit the expression of IL-2 or T-cell replacing factors' activity and serve a negative feedback function (Weiler *et al.*, 1982), while immune complexes may stimulate or inhibit the immune response (Theophilopoulos, 1980; Morgan & Weigle, 1983) by in favouring antigen receptors bridging with Fc receptors on lymphocytes (Taylor, 1982).

Few data are available concerning the turnover of retained Ig or immune complexes. Injected antibodies or antigens can displace fixed ones (Tew *et al.*, 1980; Heinen *et al.*, 1983). Antigens may detach alone or in the form of immune complexes, perhaps with the Fc receptors; this last complex can apparently influence the immune response as reviewed by Revillard & Lê Thi Bich-Thuy (1981).

Studies performed with isolated FDC cultured in the presence of various populations of lymphocytes will, perhaps, facilitate new data giving a better understanding of the phenomenona occurring in the germinal centres.

ACKNOWLEDGMENTS

We are grateful to the Fonds de la Recherche Scientifique Médicale and to the Leukaemia Research Fund for their financial support. Tonsils and adenoids were kindly provided by Dr R. Boniver (Bruyères Hospital, Liège). The authors wish to thank Dr Mason (Oxford) and Dr Stein (Kiel) for the generous gifts of monoclonal antibodies.

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