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Transfer of Antigenic Macromolecules from Macrophages to Lymphocytes

I. AUTORADIOGRAPHIC AND QUANTITATIVE STUDY OF [^{14}C]ENDOTOXIN AND [^{125}I]HAEMOCYANIN TRANSFER

C. Bona, A. Anteunis, R. Robineaux and A. Astesano

Centre de Physiologie et d'Immunologie Cellulaires, INSERM U. 104, *CNRS et Association Claude Bernard, Hôpital Saint-Antoine*, 75, *Paris XIIe, France*

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Summary. In an *in vitro* autologous system, studies were carried out on the transfer of either biosynthetically-labelled [^{14}C]*Salmonella enteritidis* endotoxin or [^{125}I]*Maia squinado* haemocyanin from macrophages to lymphocytes.

When cultured 1 hour *in vitro*, lymphocytes adhered to autologous macrophages, forming lymphocyte-macrophage islands (LMI).

Quantitative data obtained from stained thick sections showed that 1·8 per cent of the lymphocytes had adhered to macrophages with ingested [^{14}C]endotoxin while 0·6 per cent of the lymphocytes adhered to macrophages with ingested [^{125}I]haemocyanin. The presence of antigen macromolecules was observed mainly within lymphocytes adhering to macrophages with ingested antigens, i.e. at the level of LMI. On the other hand, autoradiography carried out on the same thick sections, showed that 0·20 per cent of the lymphocyte population in LMI possess silver grains. High resolution autoradiographic pictures of thin sections, showed a peculiar localization of silver grains in LMI lymphocytes: about 80 per cent of the radioactivity was found within lymphocytic nuclei and the remainder either on the cellular membrane or free in the cytoplasm. The disappearance of radioactivity from the LMI-located macrophage membranes (where lymphocytes contain silver grains) as well as the regular inhibition of antigen transfer occurring after pronase treatment of the macrophage which contained ingested antigen, strongly suggest that antigen bound to macrophage membrane was transferred to lymphocytes. As pretreatment of lymphocytes with anti-Ig serum resulted in regular inhibition of antigen transfer, it appears that the Ig receptors of lymphocyte membranes play an important role in the transfer mechanism.

Combined technique, i.e. autoradiography and the peroxidase method for revealing Ig bound to lymphocyte membranes, showed that silver grains occurred only within lymphocytes displaying peroxidase-positive membrane.

INTRODUCTION

The cooperation between macrophages and lymphocytes in the immune response has been demonstrated both *in vivo* and *in vitro* for various antigens (Fishman, 1961; Nossal, Ada and Austin, 1965; Frei, Benacerraf and Thorbecke, 1965; Gally and Feldman, 1966; Argyris, 1967; Pribnow and Silberman, 1967; Mosier, 1967; Mosier and Coppelson, 1968; Feldman, 1968; Unanue and Askonas, 1968; Dresser and Mitchison, 1968; Mitchison, 1969; Shortman, Diner, Russell and Armstrong, 1970).

The cytological and functional unit of this cooperation is probably represented by lymphomacrophagic or lymphoreticular islands. These islands have been observed on smears or sections, and also in the living state by microcinematography. Such cytological aspects were observed in the normal or immunized lymphoid organs such as bone marrow (Undritz, 1938; Berman and Stulberg, 1962), lymph nodes (Thiery, 1968a; Schoenberg, Mumaw, Moore and Weisberger, 1964; McFarland and Heilman, 1965; Hanna and Szakal, 1968; Nossal, Abbot, Mitchell and Lummus, 1968; Robineaux *et al.*, 1971), or spleen in organized cultures (Robineaux, Pinet and Kourilsky 1962). The adherence of lymphocytes to homologous macrophages, forming lymphomacrophagic islands, has also been observed *in vitro* (Fishman *et al.*, 1963; Hersh and Harris, 1968; Cline and Swett, 1968; Bona *et al.*, 1969; Hamfin and Cline, 1970; Siegel, 1970).

The macrophage lymphocyte cooperation could be considered either as:

(a) transfer of informational material either as messenger RNA (Adler, Fishman and Dray, 1966; Fishman and Adler, 1967; Adler and Fishman, 1969) or of an RNA–antigen complex (Friedman *et al.*, 1965; Askonas and Rhodes, 1965; Fishman and Adler, 1967; Bishop *et al.*, 1967; Gottlieb *et al.*, 1967; Mitshuhashi, Kurashiga, Kawakami and Nogimar, 1968; Bona, 1971a; Paque and Dray, 1971) from the macrophages to the lymphocytes.

(b) direct contact between antigen bound to macrophage membrane and lymphocytes (Unanue, Cerottini and Bedford, 1969; Unanue and Cerottini, 1970).

(c) transfer of macromolecules or of antigenic fragments. Findings on this last point are contradictory. Some authors recovered the antigen within lymphoid cells, after *in vivo* administration (Roberts and Haurowitz, 1962; Wellensick and Coons, 1964; Nossal *et al.*, 1968; Conrad and Demoise, 1970; Mitchell and Abbot, 1971); whereas most authors have only shown a concentration of antigen in the macrophages of the lymphoid organs without having recovered it in the antibody-forming cells (Cohen *et al.*, 1965; Mitchell and Abbot, 1965; Balfour and Humphrey, 1966; McDevitt *et al.*, 1966; Humphrey and Frank, 1967; Hana and Szakal, 1968; Humphrey, 1969).

In order to elucidate the mechanism through which macrophages can inform lymphocytes in the immune response, we studied the transfer of antigen from macrophages to lymphocytes in an *in vitro* autologous system.

We chose the *in vitro* system because of several advantages as compared to the *in vivo* system:

1. the possibility of concentrating within a short lapse of time a large quantity of antigens into the macrophages. This point was essential in Aronson's experiment (1963) demonstrating microbe transfer between hystiocytes. Furthermore, the concentration of *in vivo* injected antigen is always low because of its dilution in the body.

2. the possibility of dissociating the phenomenon into two steps; the uptake of the antigen by the macrophages, followed by incubation with lymphocytes. *In vivo*, this phenomenon cannot be dissociated because the antigen carried by the blood flow comes into contact with both the cellular types almost simultaneously.

3. the possibility of following the uptake and the transfer of the antigen in the absence of antibody. When the antigen is administered *in vivo*, it is difficult to state whether the accumulation in macrophages represents one of the steps of antibody formation, or whether it follows this formation (Nossal, 1965; Humphrey and Frank, 1967).

4. Finally, this type of two-step experiments enabled us to study the influence of different factors on macrophage or lymphocyte membrane during the transfer process.

Using biosynthetically-labelled [^{14}C]endotoxin, and [^{125}I]labelled haemocyanin, we observed their transfer from guinea-pig macrophages to autologous lymphocytes. This

transfer is inhibited by pronase treatment of the macrophages with ingested antigen or by pre-treatment of the lymphocytes with anti-guinea-pig immunoglobulin serum. Lymphocytes containing the antigen show immunoglobulins on their surface.

MATERIALS AND METHODS

1. *Cells*

(a) *Macrophages.* Normal guinea-pig peritoneal exudate was obtained by injecting 15 ml of mineral oil (Merck). Three days after injection, the cells from the peritoneal exudate were harvested by washing the peritoneum with a heparinized isotonic saline solution. The cells (10^7/ml) were cultured for 2 hours in Falcon flasks in medium 199 supplemented with 5 per cent heat inactivated calf serum (Pasteur Institute) under an atmosphere of 5 per cent CO_2. After 2 hours, the supernatant containing the non-adherent cells was decanted. The layer of adherent cells showed 98 per cent of macrophages by Wright staining.

(b) *Lymphocytes.* The autologous lymphocytes were isolated from inguinal, popliteal, axillary and retro-scapular lymph nodes. The lymph nodes were chopped and teased through an 80-mesh stainless steel sieve. The cells thus collected, showed no agglutination by phase contrast microscopy.

Similar cells were collected from primed guinea-pigs which had been injected in the footpad with either 10 μg of *Salmonella enteritidis* endotoxin, or with 15 μg of *Maia squinado* haemocyanin. The cells were harvested 60 days after injection of endotoxin and 35 days after injection of haemocyanin.

2. *Antigens*

S. enteritidis endotoxin, extracted by a phenol-water method, was biosynthetically labelled with ^{14}C (specific activity 11·2 μCi/mg) according to Ribi *et al.* (1961).

Maia squinado haemocyanin (Calbiochem) was labelled with ^{125}I (specific activity 0·25 μCi/μg) according to Greenwood *et al.* (1963).

3. *Transfer system*

The macrophage monolayer (10^7 cells) was put in contact for 1 hour at 37° with the labelled antigen in 10 ml of medium 199 supplemented with 5 per cent heat inactivated calf serum. To the macrophage layer was added either 150 μg/10^7 cells of [^{14}C]endotoxin, or 100 μg/10^7 of [^{125}I]haemocyanin. After incubation the cell layer was washed five times in the same medium and 5×10^8 autologous lymphocytes were added. After 1 hour at 37°, the supernatant was carefully decanted and the cell layer fixed for 1 hour at 4° with 1·5 per cent buffered glutaraldehyde. This layer was then collected and embedded for electron microscopy.

The following controls were used:

(a) macrophages incubated simultaneously with the same labelled antigen but glutaraldehyde instead of medium 199, washed five times and later put in contact with the lymphocytes in the culture medium.

(b) 'L' fibroblasts incubated with the same labelled antigen and put in contact with the lymphocytes.

With endotoxin, two additional controls were performed:

(a) the transfer system as above but incubated at 4°.

(b) supernatant fraction from macrophage antigen incubation in the absence of lympho-

cytes was subsequently added to the lymphocyte populations for 1 hour at 37°. This control was performed to determine whether the antigen excreted from macrophages could account for the positive autoradiographic reaction within lymphocytes.

4. *Cells treated by different agents*

(a) *Macrophages.* The macrophages after incubation with antigen, before contact with the lymphocytes, were incubated in medium 199 containing either 100 μg/ml RNase (Calbiochem) for 30 minutes at 37°, or 1 mg/ml pronase (Calbiochem) for 1 hour at 37°.

(b) *Lymphocytes.* For similar experiments, some lymphocyte samples (10^8/ml) were treated for 30 minutes at 37° with 0·2 ml of rabbit anti-guinea-pig immunoglobulin serum (Pasteur Institute) and washed three times in medium 199 prior to contact with macrophages.

5. *Autoradiography techniques*

After embedding in epon, thick and thin sections, homogeneous in colour, were cut on an ultratome LKB III and mounted on glass slides.

(a) *Autoradiography of thick sections*: Thick sections mounted on glass slides were coated by dipping in K5 emulsion (Ilford) diluted 1:2 in distilled water. After exposure for 8 or 12 days, the slides were developed for 4 minutes in D 19 Kodak at 18°, and fixed for 10 minutes in 30 per cent hyposulphite.

The slides were stained at 37° with filtered, 0·5 per cent toluidine blue in 1 per cent sodium borate for a few seconds, and washed in 70 per cent alcohol. After drying, the slides were mounted in DPX for light microscopy.

(b) *High resolution autoradiography.* The electron microscope sections (yellowish-white colour) were mounted on celloidin-coated slides. These sections were stained with uranyl acetate and lead citrate, and then covered with a carbon film. Radioautographic exposure was then performed by dipping the slides into L4 emulsion (Ilford) diluted 1:5 in distilled water. The exposure times were 6–8 weeks. After development in Kodak microdol X, the sections were stripped from the glass and mounted on grids. The grids were examined with an Elmiskop I (Siemens).

6. *Immunochemical techniques*

After the transfer experiments, some of the samples were put in contact, prior to fixation, with rabbit anti-guinea-pig immunoglobulin serum for 30 minutes at 37° (10^8 cells/ml with 0·5 ml serum diluted 1:4). After three washes in PBS, the cells were incubated with peroxidase-labelled sheep anti-rabbit IgG serum (diluted 1:10), according to Avrameas (1968). After another three washes, the cells were fixed for 15 minutes in cold 1·5 per cent phosphate-buffered glutaraldehyde, washed again three times and incubated for peroxidase, according to the method of Graham and Karnovsky (1966). After two washes, the cells were left for 18 hours in a monodiphosphate buffer containing 5 per cent sucrose, and prepared for high resolution autoradiography

Controls consisted of replacing on the one hand the rabbit anti-guinea-pig IgG serum by normal rabbit serum (immunological control), and on the other hand replacing the labelled peroxidase serum by a free peroxidase (cyto-chemical control). Another cyto-chemical control for peroxidase technique was performed: the cells were incubated in Graham medium supplemented with 0·1 M KCN.

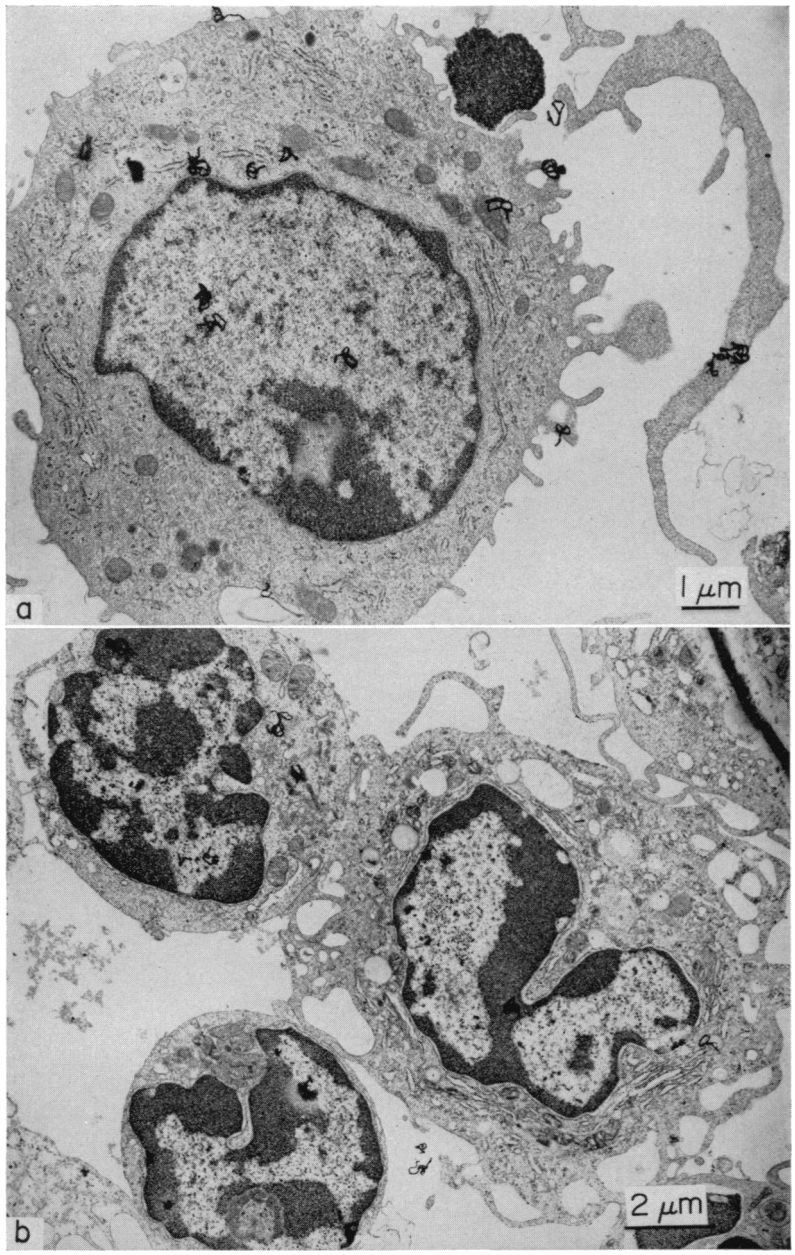

FIG. 1. High resolution radiographic aspects of the transfer of [^{14}C]endotoxin from macrophages to lymphocytes at LMI level. (a) Macrophages after 2 hours of contact with [^{14}C]endotoxin showing numerous silver grains on the cellular surface. (b) Note the presence of silver grains within macrophages and one lymphocyte at LMI level formed by three lymphocytes adhering to a macrophage.

TABLE 1

THE FORMATION OF LYMPHO-MACROPHAGE ISLANDS (LMI) AND THE TRANSFER OF LABELLED ANTIGEN

Cellular interaction types	Per cent lymphocytes within LMI	Per cent labelled lymphocytes within LMI	Per cent labelled cells within LMI		Per cent of free labelled lymphocytes	Total number of lymphocytes counted
			Macrophages	Lymphocytes		
Macrophages ingested [^{14}C]endotoxin + Lymphocytes	1·8	0·2	100	10·1	0·2	13,664
RNase treated macrophages ingested [^{14}C]endotoxin + Lymphocytes	3·8	0·4	100	9·5	0·9	3,923
Pronase treated macrophages ingested [^{14}C]endotoxin + Lymphocytes	3·1	0	100	0	0·1	5,088
Macrophages ingested [^{14}C]endotoxin + Ig serum pretreated lymphocytes	1·1	0	100	0	0·1	3,575
Macrophages incubated simultaneously with glutaraldehyde+ [^{14}C]endotoxin + Lymphocytes (control)	0	0	0	0	0	12,000
'L' fibroblasts ingested [^{14}C]endotoxin + Lymphocytes (control)	2·1	0	100	0	0·04	1,700

TABLE 1 (continued)

Cellular interaction types	Per cent lymphocytes within LMI	Per cent labelled lymphocytes within LMI	Per cent labelled cells within LMI		Per cent of free labelled lymphocytes	Total number of lymphocytes counted
			Macrophages	Lymphocytes		
Macrophages ingested [^{14}C]endotoxin + Lymphocytes (4°-incubation)	0·5	0	100	0	0·01	27,000
Culture medium of 1 hour macrophages ingested [^{14}C]endotoxin + Lymphocytes	0	0	0	0	0·009	18,000
Macrophages ingested [^{125}I]Hcy + Lymphocytes	0·6	0·2	100	32	0·2	11,739
RNase treated macrophages ingested [^{125}I]Hcy + Lymphocytes	0·8	0·2	100	25	0·12	8,903
Pronase treated macrophages ingested [^{125}I]Hcy + Lymphocytes	0·2	0·005	100	3	0·05	12,225
Macrophages ingested [^{125}I]Hcy + anti-Ig serum pretreated lymphocytes	1·6	0·03	100	1·8	0·3	6,618
Macrophages incubated simultaneously with glutaraldehyde + [^{125}I]Hcy + Lymphocytes (control)	0	0	0	0	0	12,400
'L' fibroblasts ingested [^{125}I]Hcy + Lymphocytes (control)	2·1	0	100	0	0·05	1,700

RESULTS

1. [^{14}C]ENDOTOXIN TRANSFER

(A) *Ultrastructural aspects of transfer*

After contact with [^{14}C]endotoxin, the autoradiographs showed the following pattern of silver grains in macrophages:

—about 1/3 of the silver grains were observed at the cell surface of the macrophages or on their cytoplasmic processes

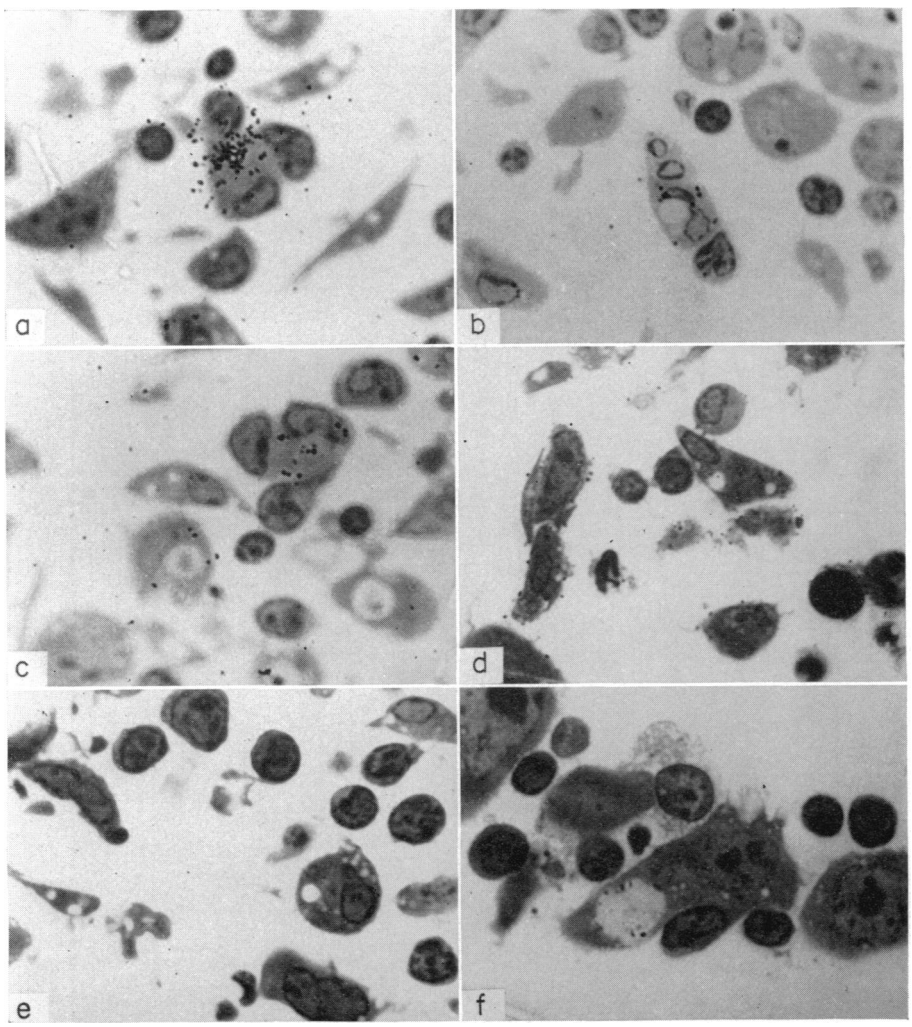

FIG. 2. Radioautographic aspect of thick sections of the transfer of [^{14}C]endotoxin. (a) Macrophages ingested [^{14}C]endotoxin—put in contact with autologous lymphocytes. (b) RNase-treated macrophages ingested [^{14}C]endotoxin—put in contact with autologous lymphocytes. (c) Pronase-treated macrophage ingested [^{14}C]endotoxin—put in contact with autologous lymphocytes. (d) Macrophages ingested [^{14}C]endotoxin—put in contact with rabbit anti-guinea-pig Ig serum-pretreated autologous lymphocytes. (e) Control. Macrophages incubated simultaneously with glutaraldehyde and [^{14}C]endotoxin put in contact with autologous lymphocytes. (f) Control. 'L' fibroblasts ingested [^{14}C]endotoxin put in contact with guinea-pig lymphocytes.

—about 2/3 were found within the cytoplasm. In the cytoplasm, the silver grains were localized either at the vacuole level, or free in the cytoplasm near the endoplasmic reticulum, or in the ribosome-rich regions (Fig 1a).

Lymphocytes incubated with macrophages which had ingested antigen adhered to the macrophages in small numbers. The structures composed of one macrophage, and a minimum of two adhering lymphocytes, were considered to be lymphocyte-macrophage islands (LMI). We did not find either plasmocytes or any other cell type in these associations, nor did we observe any intercellular bridges between these two cell types. At the level of some LMI, we observed the presence of silver grains both in the macrophages and in the lymphocytes.

In the cytoplasm of LMI macrophages, the number of silver grains was smaller than in free macrophages; moreover, they could not be found on the membrane. In the LMI lymphocytes, we observed between 3 and 9 grains with an average of 5; some of those grains were found in the cytoplasm, but 80 per cent were recovered in the nucleus (Fig. 1b).

(B) *Quantitative aspect of the transfer*

In our experimental system, 1·8 per cent of the lymphocytes were recovered forming LMI, but only 0·2 per cent showed silver grains in the two types of cells (positive LMI). This indicated that a transfer of labelled macromolecules occurs in only 10 per cent of the lymphocytes recovered in the positive LMI.

Controls for this experiment were negative. When [^{14}C]endotoxin was put in contact with the macrophages simultaneously with glutaraldehyde, the lymphocytes adhered to the macrophages, albeit in reduced number, but no transfer occurred. When macrophages were replaced by fibroblasts, 2·1 per cent of lymphocytes adhered to the latter, but they did not contain any silver grains. Also, when the transfer was performed at 4° or lymphocytes were incubated with the supernatant fraction of the culture medium of macrophages which had ingested antigen, the lymphocytes did not possess silver grains.

(C) *Influence of various factors on the transfer*

Treatment by RNase or by pronase of macrophages with ingested [^{14}C]endotoxin increased the number of LMI to 3·8 per cent for RNase and 3·1 per cent for pronase. In the RNase treated macrophages, only 0·4 per cent of the 3·8 per cent LMI were positive, whereas after pronase treatment all LMI were negative. Pre-treatment of lymphocytes with rabbit anti-guinea-pig Ig serum prior to their contact with the macrophages with ingested-labelled endotoxin also inhibited the transfer. These results are presented in Table 1 and illustrated in Fig. 2a,b,c,d,e,f.

(D) *Immunocytochemical data*

Cells from endotoxin primed guinea-pigs were used for transfer experiments under the same conditions as cells from non primed animals. After the transfer, the cells of immunized animals were treated with rabbit anti-guinea-pig Ig serum and with peroxidase labelled-sheep anti-rabbit IgG serum. We did not observe a peroxidase reaction on the surface of lymphocytes containing silver grains.

2. TRANSFER OF [^{125}I]HAEMOCYANIN

(A) *Ultrastructural aspect*

After contact with [^{125}I]haemocyanin, the silver grains in the macrophages were

distributed either on the cell surface or in the cytoplasm. In the cytoplasm, the grains were observed either in endocytotic vacuoles or in secondary lysosomes, or else free, in the ribosome-rich regions.

The lymphocytes put in contact with these macrophages adhere to them forming LMI in which we observed the presence of silver grains in both cellular types. In the lymphocytes,

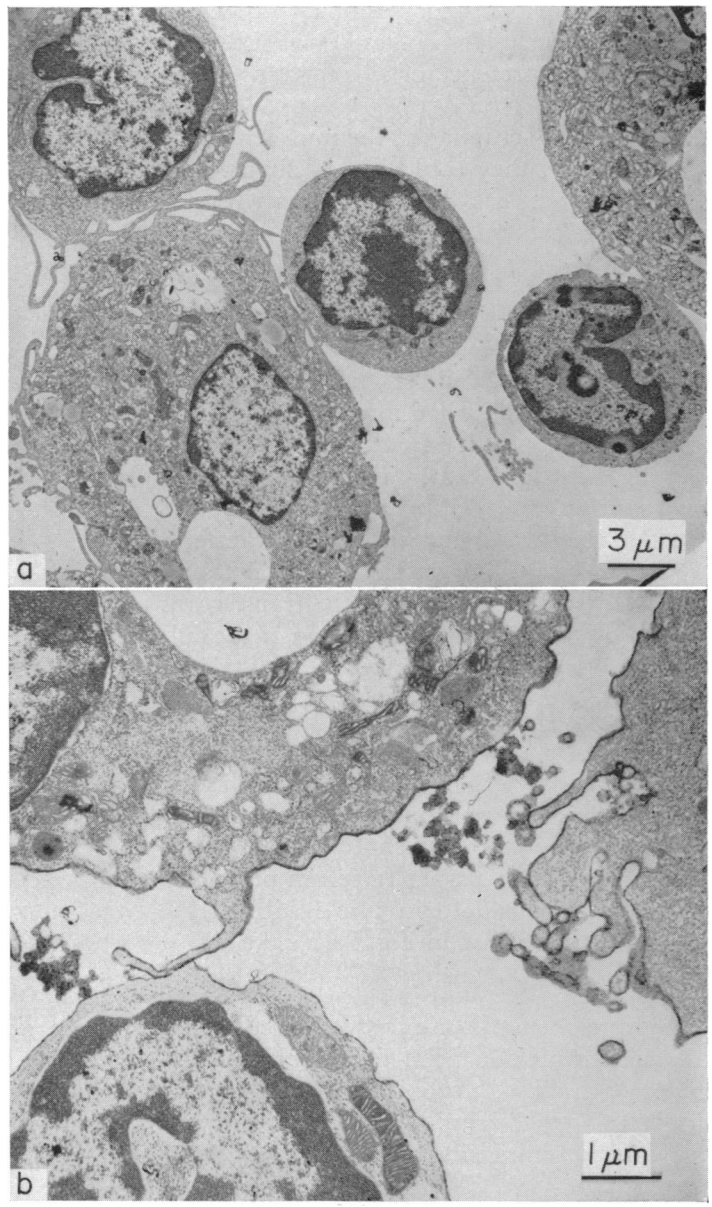

FIG. 3. High resolution radioautographic aspects of the transfer of [^{125}I]haemocyanin at LMI level. (a) Note the presence of silver grain within macrophages and lymphocytes at LMI level. (b) Silver grains are seen within macrophages and lymphocytes showing peroxidase positive reaction at their surface.

the grains were located either at the cell surface, or in the cytoplasm, but also in the nucleus (Fig. 3a).

(B) *Quantitative aspect of the transfer*

Autologous lymphocytes adhere to the macrophages forming LMI in a proportion of 0·6 per cent among which 0·2 per cent showed silver grains in both cellular types. This means that a transfer occurs in some 30 per cent of the LMI lymphocytes. The controls, as for the endotoxin experiments, were negative.

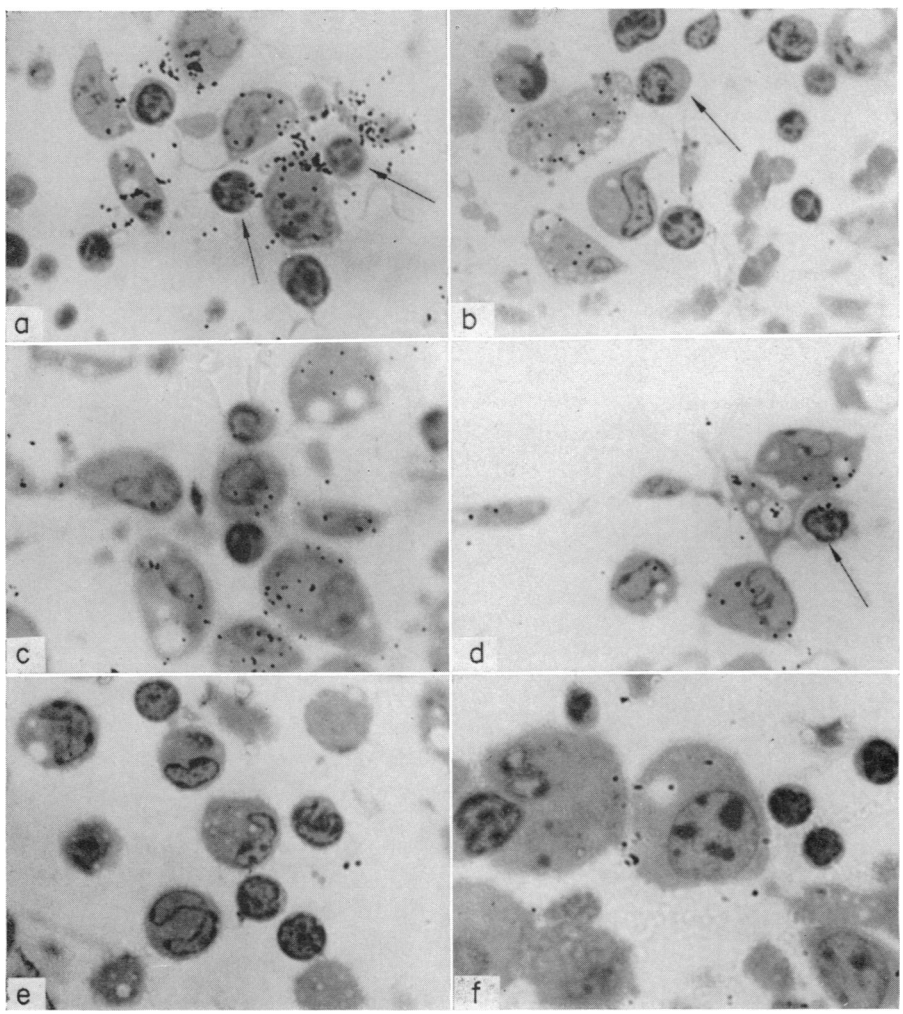

FIG. 4. Radioautographic aspects of thick sections of the transfer of [^{125}I]haemocyanin. (a) Macrophages ingested [^{125}I]haemocyanin put in contact with autologous lymphocytes. (b) RNase-treated macrophages ingested [^{125}I]haemocyanin put in contact with autologous lymphocytes. (c) Pronase-treated macrophages ingested [^{125}I]haemocyanin put in contact with autologous lymphocytes. (d) Macrophages ingested [^{125}I]haemocyanin put in contact with rabbit anti-guinea-pig Ig serum-pretreated autologous lymphocytes. (e) Control. Macrophages incubated simultaneously with glutaraldehyde and [^{125}I]haemocyanin put in contact with autologous lymphocytes. (f) Control. Fibroblast ingest [^{125}I]haemocyanin with guinea-pig lymphocytes.

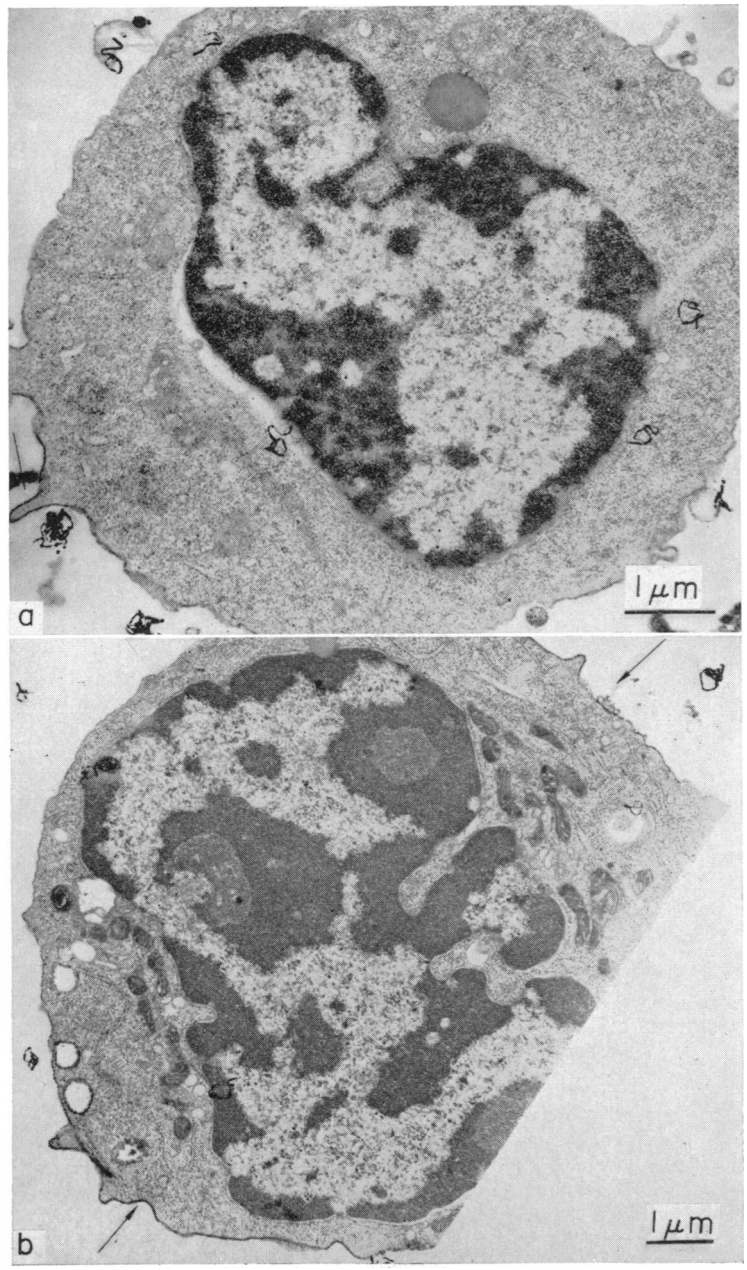

FIG. 5. High resolution radioautographic aspects of lymphoid cell from LMI (haemocyanin experiments). (a) Lymphoblast cell showing numerous silver grains and discontinuous peroxidase positive reaction at the membrane level (←). (b) Immunoblast showing numerous silver grains and peroxidase positive reaction at their membrane (←).

(C) *Influence of various factors on the transfer*

Treatment with RNase of macrophages with ingested [^{125}I]haemocyanin did not change the incidence of positive LMI, although the percentage of lymphocytes adhering to macrophages increased (0·8 per cent) as compared to non-treated macrophages.

Treatment of macrophages with pronase considerably decreased the number of positive LMI (0·005 per cent) and also decreased the lymphocyte-macrophage associations (1·6 per cent). Pre-treatment of lymphocytes with rabbit anti-guinea-pig Ig serum greatly decreased the transfer. The number of positive LMI was only 0·03 per cent.

These results are presented in Table 1 and illustrated in Fig. 4a,b,c,d,e,f.

(D) *Immunocytochemical data*

Cells from immunized guinea-pigs were treated after the transfer with rabbit anti-guinea-pig Ig serum and then with peroxidase-labelled sheep anti-rabbit IgG serum.

High resolution autoradiography and immunocytochemical observations showed that lymphocytes constituting the LMI contained silver grains (positive transfer) and also gave a positive peroxidase reaction on their surface (Fig. 3b).

There were more silver grains in the blast cells than in the small lymphocytes. In these cells, the peroxidase reaction was discontinuous and a considerable number of silver grains could be observed on their surface (Fig. 5a,b).

The lymphocytes forming LMI, but with a negative peroxidase reaction, did not contain any silver grains.

DISCUSSION

In bacteria, the transfer of genetic material occurs either directly by conjugation, or, in the conversion phenomenon, carried by a phage.

In eukaryotes, there are only a few reports concerning the passage of substances from one cell to another: the passage of vital stains between the epithelial cells of Drosophila salivary gland (Kanno and Lowenstein, 1966), the passage of bacteria between histiocytes (Aronson, 1963), the transfer of DNA between leukaemia lymphocytes and 'L' fibroblasts of the mouse (Hill and Spurna, 1968), and the RNA from macrophage to lymphocytes (Fishman *et al.*, 1963; Bona *et al.*, 1969; Paque and Dray, 1970).

Sharp and Burwell (1960) suggested that at the level of macrophage islands, observed in the spleen and lymph nodes of immunized animals, transfer of antigen in the spleen and lymph nodes of immunized animals, could take place from macrophages to antibody-forming cells.

In our experiments, with two differently labelled antigens we are able to observe the transfer of antigens from macrophages to lymphocytes. The percentage of lymphocytes constituting the LMI was 0·6 per cent for haemocyanin, and 1·8 per cent for endotoxin. The percentage of positive LMI for both antigens was 0·2.

The presence of the silver grains within the lymphoid cells after *in vitro* administration of radioactive antigens was reported by several authors (Nossal *et al.*, 1968, Mitchell and Abbot, 1971). In this type of '*in vitro*' experiment of course it could not be ascertained whether the antigen recovered in the lymphocytes had been directly taken up by these cells or via the macrophages.

The statistics performed by us on thick sections showed clearly that the labelling of lymphocytes adhering to macrophages occurs in a proportion of 0·2 per cent. The absence

of all traces of radioactivity in lymphocytes when macrophages are replaced by fibroblasts in the same conditions proves that the transfer of the antigen only occurs at the LMI level.

Free-labelled lymphocytes (not adhering to macrophages) were found in a proportion of 0·2 per cent in the endotoxin experiments as well as with haemocyanin. This labelling can be explained as follows: lymphocytes might be released from the LMI during various manipulations, eight washings + electron microscope technique or else the free antigen could be directly taken up by these cells.

We deem the first hypothesis to be more likely: by phase contrast, five to eight lymphocytes were found within LMI. One should also recall that the microcinematographic dynamic study of LMI islands showed that lymphocytes can spontaneously leave an island (Robineaux *et al.*, 1962). The fact that the free lymphocytes were not labelled in the control experiments, i.e. when the antigen had been added to the macrophages simultaneously with glutaraldehyde or when the lymphocytes were incubated in the same culturc medium provided from macrophages with ingested antigen, suggested that the possibly free antigen or fragments of broken down antigen liberated from macrophages are not taken up by the free lymphocytes.

Our findings suggest that antigen bound to the macrophage membrane represents the transferable fraction of the antigen taken up by macrophages. Actually, we observed that in positive LMI, macrophages no longer have silver grains on their surface and on the other hand pronase treatment of the macrophage with ingested antigen inhibited the transfer, although numerous grains were present in their cytoplasm. It is known that macrophage membrane bound antigen is considerably more effective than in the free form (Unanue *et al.*, 1969, Unanue and Cerottini, 1970; Kölsch, personal communication). It should also be noted that the transfer of the antigen bound to membrane does not take place through membrane RNA. The presence of the RNA in the membrane of various cells was reported by Weiss *et al.* (1962) and recently in rabbit alveolar macrophages by Nachman *et al.* (1971). As mentioned, RNase treatment of macrophages with ingested antigen does not inhibit the transfer of antigen carried by macrophage RNA, albeit different from the RNA membrane, since several authors succeeded in inducing an immune response with RNA extracted from macrophage-associated antigen (Fishman, 1965; Fishman *et al.*, 1963; Friedman *et al.*, 1965; Bishop *et al.*, 1967; Askonas and Rhodes, 1965; Gottlieb *et al.*, 1967; Mitshushash *et al.*, 1968; Paque and Dray, 1970; Bona, 1971a).

The IgG from the lymphocyte surface might also play an active role. Indeed, pretreatment of lymphocytes with rabbit anti-guinea-pig Ig serum, prior to contact with macrophages, inhibits the transfer.

Using cells from the haemocyanin primed animals, we observed that only those lymphocytes which exhibited silver grains possessed IgG on their surface.

These results suggest that the presence of IgG on the lymphocyte surface could not only play a part in the recognition of the antigen bound to macrophages, but also in the transfer. However, with cells from endotoxin immunized animals, we were unable to show the presence of IgG using the same immunocytochemical method. This could be explained by the IgM-type immune response which is characteristic of endotoxins (Britton and Moller, 1966).

Our data cannot establish the thymus or marrow origin of the lymphocytes which adhere to macrophages with ingested antigen and exhibit silver grains. It is stressed now that thymus lymphocytes have also been shown to possess Ig receptors on their surface (Greaves, 1970).

What is the meaning of the radioactivity observed in lymphocytes at the LMI level?

Ehrenreich and Cohn (1967) have shown that the main product excreted by macrophages after uptake and breakdown of bovine serum albumin [^{125}I]was iodotyrosine. In the case of haemocyanin, it is possible that the radioactivity observed in lymphocytes represents the degradation products of this antigen. In the case of endotoxin, where labelling was performed biosynthetically, the silver grains could correspond to the whole antigen or to antigen fragments. It has been shown that 48 hours after uptake of endotoxin by guinea-pig macrophages, their ability to induce the Schwartzman phenomenon, elicit an immune response, or sensitize red cells, is not destroyed (Mesrobeanu *et al.*, 1969; Bona, 1971a).

In addition, when the lymphocytes were incubated with 1-hour culture medium of macrophages which had ingested [^{14}C]endotoxin, the number of lymphocytes containing silver grains was non significant (0·01 per cent).

The intimate mechanism of this transfer can be explained in two ways: the antigen can penetrate within lymphocytes, either by uptake of the antigen fraction bound to the macrophage surface (1/3 of the observed grains), or can be actively transferred from macrophages to lymphocytes. The uptake of antigen macromolecules by a micropinocytosis mechanism has been observed in lymphocytes (Thiery, 1968b; Han and Johnson, 1966; Robineaux *et al.*, 1969). The fact that the grains are free in the cytoplasm could indicate that the antigen can directly pass through the cell membrane, as has already been considered by several authors (Moore *et al.*, 1961; Florey, 1967; Nossal *et al.*, 1968). Schoenberg *et al.* (1964) observed intercellular bridges containing ribosomes between the plasmocytes and/or the lymphocytes and macrophages. This type of intercellular contact would easily explain the transfer, but throughout our experiments we never encountered intercellular lympho-macrophage bridges. The hypothesis of penetration through micropinocytosis cannot be excluded, even if the labelled material were free in the cytoplasm. It is indeed known that the micropinocytotic vacuoles are finally reabsorbed.

The immunological meaning of this transfer still remains to be worked out but preliminary results suggest that it is related to antibody synthesis.

In the same experimental system, the lymphocytes were incubated for 24–72 hours with macrophages with ingested endotoxin. The presence of specific antibodies on the surface of lymphocytes and secreted antibodies was investigated by several methods. The specific antibodies on the surface of lymphocytes were visualized either by the rosette method using endotoxin sensitized erythrocytes or antigen binding of [^{14}C]endotoxin using an autoradiographic technique.

All these techniques showed that the transfer of endotoxin from macrophage to lymphocytes has an immunological significance.

The last question concerned the significance of the nuclear radioactivity:

After a 1-hour contact between macrophages with ingested antigen and lymphocytes forming positive LMI, the silver grains were observed in the lymphocytes at the plasma membrane level, in the cytoplasm and also in the nucleus.

Nossal *et al.* (1968) and Mitchell and Abbot (1971) have observed the presence of silver grains in the nuclei of lymphocytes from lymph nodes or spleens of mice inoculated *in vivo* with radioactive flagellin. These authors tried to explain the presence of silver grains within the nucleus of lymphocytes as follows:

(a) the silver grains observed in the nucleus of lymphocytes could correspond to isotopes dragged by the knife edges from their true *in vivo* location during the preparation of the

sections. Although such an explanation might be taken into account in the case of radioactive ions like ^{125}Iodine or ^{131}Iodine released from the antigen by the dehalogenases present in various organs (Tata, 1960), such a possibility seems quite unlikely with a biosynthetic label.

In addition, recent biochemical studies performed by Willerson *et al.* (1971) have shown that after *in vivo* administration of [^{14}C]endotoxin, the antigen is recovered only in the nuclear and lysosomal fractions of rat or mouse spleen cells.

(b) the high energy emissions of ^{125}I, or the Auger electrons might take an oblique path in relation to the initial source. If this were valid, it is difficult to understand how 3–9 silver grains could be recovered in the lymphocytes when the background is negligible (2–3 grains per square of grid). Moreover, in the case of ^{14}C, the strong β-rays are not accompanied by Auger electrons.

(c) the absence of the silver grains in thin serial sections reported by Mitchell and Abbot (1971) could not represent strong evidence in favour of random labelling of the lymphocyte nucleus after in vivo administration of radioactive antigen. The thin sections were about 600 ± 100 Å and the size of flagellin is only a few Angstroms; consequently the absence of silver grains in serial thin sections does not account for absence of antigen in nucleus.

The statistics of the distribution of silver grains within the lymphocyte nucleus at LMI level, corrected by eradication of the resolution error of Ilford L4 emulsion and strong energetic ^{14}C β-particles permitted us to establish that 12·6 per cent of the silver grains found in the lymphocytes were localized within the nucleus (Bona, 1971b).

On the other hand, it is quite satisfactory to associate the presence of the antigen in the nucleus, with the fact that the first synthesis of the antibody appears in the perinuclear space (Leduc *et al.*, 1969). The mechanism of penetration of the macromolecules into the nucleus is still hardly known but seems cytologically possible (Robineaux, 1964).

The derepression mechanism for the structural genes responsible for the autologous polypeptide chain synthesis is unknown; however according to Szilard (1960), the antigen can act as derepressor, or, as a 'template' according to Haurowitz (1969).

A possible relationship between the rapid penetration of the antigen into the nucleus and the mechanism of the derepression of the structural genes can only be conjectured at this time.

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