

The mode of interaction with macrophages of two ordered synthetic polypeptides which differ in their thymus dependency

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Summary. The mode of interaction with macrophages of two ordered synthetic polypeptides (Tyr-Tyr-Glu-Glu)-poly(DLAla)—poly(Lys), (T-T-G-G)-A—L, and (Tyr-Glu-Tyr-Glu)-poly(DLAla)—poly(Lys), (T-G-T-G)-A—L, which differ in their requirements for T-B cell co-operation in the process of antibody production, was compared. The binding of the two radiolabelled antigens to the surface of peritoneal adherent cells, their uptake by the cells and the rate of their degradation were investigated. Macrophages were found to be capable of degrading both polypeptides with the same efficiency. (T-G-T-G)-A—L, the antigen which is less T-dependent, was bound to macrophage surfaces more readily than (T-T-G-G)-

A—L, the T-dependent antigen, however, its uptake by the cells was found to be lower. Thus, (T-G-T-G)-A—L remains for a longer period in the form of a membrane bound polyvalent antigen.

INTRODUCTION

Previous reports have shown that the immune responses towards two ordered polypeptides (Tyr-Tyr-Glu-Glu)-poly(DLAla)—poly(Lys), designated (T-T-G-G)-A—L, and (Tyr-Glu-Tyr-Glu)-poly(DLAla)—poly(Lys), denoted (T-G-T-G)-A—L, which are composed of the same amino acids and differ only in their order, are under different genetic controls (Mozes, Schwartz & Sela, 1974; Schwartz, Mozes & Sela, 1975). Furthermore, these two ordered polypeptides differ in their requirement for T-B cell co-operation in the process of induction of antibody production (Schwartz, Hooghe, Mozes & Sela, 1976). Thus, (T-T-G-G)-A—L is a T-dependent antigen, while (T-G-T-G)-A—L can activate B cells to mount a specific immune response without T-cell help or with only minor dependency on T-cells (Schwartz *et al.*, 1976). It has been shown for other antigenic systems that differences in the optical configuration of the amino acids which compose the antigens might be responsible for their different capability to trigger B cells. Thus, the two multi-chain polypeptide antigens poly(LTyr, LGlu)-poly

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Abbreviations: (T,G)-A—L, poly(LTyr, LGlu)-poly(DLAla)—poly(LLys); (T-T-G-G)-A—L, (Tyr-Tyr-Glu-Glu)-poly(DLAla)—poly(LLys); (T-G-T-G)-A—L, (Tyr-Glu-Tyr-Glu)-poly(DLAla)—poly(LLys); T, thymus-derived; B, bone marrow-derived.

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(LPro)—poly(LLys) and poly(DTyr, DGlu)-poly(DPro)—poly(DLys) which differ only in the optical configuration of the amino acids of which they are composed are T-dependent and T-independent, respectively (Sela, Mozes & Shearer, 1972).

The precise molecular requirements for thymus independency have not yet been elucidated. However, it was suggested that the presence of repeating antigenic determinants (Sjöberg & Möller, 1970; Feldman & Basten, 1971) as well as slow rate of degradation (Sela *et al.*, 1972) are part of the prerequisites for T-independency. It seems plausible that multivalency with respect to specific antigenic determinants is not sufficient for triggering B cells without the requirement for T-cell help but that the polymer has to remain in the body in its polymeric undegraded form for long periods (Bell, 1975). Thus, the different modes of responses towards poly(DTyr, DGlu)-poly(DPro)—poly(DLys), poly(DTyr, DGlu)-poly(LPro)—poly(LLys) and poly(LTyr, LGlu)-poly(LPro)—poly(LLys) may result from the long survival of the first two polymers in the body (Medlin, Humphrey & Sela, 1970) either free or bound to the surface of cells such as macrophages (Sela *et al.*, 1972).

In the present study we have compared the interaction of the two ordered polypeptides (T-T-G-G)-A—L and (T-G-T-G)-A—L with macrophages. Our working hypothesis was that the thymus independency of (T-G-T-G)-A—L may stem from differences in the specific handling of the two antigens by macrophages. Therefore, the binding of the two antigens to the surface of macrophages, their uptake by these cells and the rate of their subsequent degradation were investigated. It appears that macrophages are capable of degrading (T-G-T-G)-A—L as well as (T-T-G-G)-A—L. However, although the former antigen is bound to the cells more efficiently, its uptake by macrophages is lower than that of (T-T-G-G)-A—L. It is likely, therefore, that (T-G-T-G)-A—L persists in a polyvalent form for a longer period on the surface of macrophages.

MATERIALS AND METHODS

Animals

C3H.SW mice were obtained from the Experimental Animal Unit of the Weizmann Institute of Science.

Antigens

The antigens used in this study were the (LTyr-LGlu-LGlu)-poly(DLAla)—poly(LLys), abbrevi-

ated (T-T-G-G)-A—L, (LTyr-LGlu-LTyr-LGlu)-poly(DLAla)—poly(LLys) designated (T-G-T-G)-A—L as well as the random polypeptide poly(LTyr, LGlu)-poly(DLAla)—poly(LLys), abbreviated (T,G)-A—L. The synthesis and the characterization of these immunogens were previously described (Mozes *et al.*, 1974; Schwartz *et al.*, 1975; Sela, Fuchs & Arnon, 1962).

Immunization

Antisera specific for the antigens were obtained by immunization of mice with 10 μ g of the antigen in 0.06 ml of Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, Mich.), injected into the hind footpads, intradermally. Three weeks later a booster injection with the same antigen in PBS was given. The mice were bled 10 days after the second injection.

Iodination

(T-T-G-G)-A—L and (T-G-T-G)-A—L were labelled with 125 I (carrier free) according to the method of Greenwood, Hunter & Glover (1963) using 1 mCi of 125 I for 100 μ g of protein. Free iodine was removed either by gel filtration, using Sephadex G-25 or by dialysis against PBS.

Liposomes

Liposomes were prepared according to Smolarsky, Teitelbaum, Sela & Gitler (1977). A solution in chloroform:methanol (1:1) of phosphatidylcholine, cholesterol, dicetylphosphate and α -tocopherol in the molar ratios of 6.0:4.3:0.15:0.77 was prepared (10 mg total lipids). The solution was evaporated under nitrogen and dried under vacuum for several hours. To the dried material 1 ml of solution of iodinated antigen was added and kept at room temperature for 3–4 h. It was then suspended by a vortex and the separation of liposomes from the nontrapped material was performed, by chromatography on Sepharose 4B. The liposomes were eluted in the void volume and stored under nitrogen until used.

Macrophages

Peritoneal cells were harvested from normal mice and from mice stimulated intraperitoneally (i.p.) with 2 ml of 3% thioglycollate (Difco, Detroit, Mich.). The total number of cells obtained from normal mice was about $4 \times 10^6 - 6 \times 10^6$, 30–40% of which were macrophages. Four days after thioglycollate stimulation, about 30×10^6 cells were

obtained. More than 80% of these cells were macrophages.

Cultivation of macrophages

Macrophage cultures were prepared as described by Bar-Eli & Gallily (1975). Unstimulated and thiolglycollate stimulated cells were washed either with Minimal essential medium (MEM) or with Hanks's balanced salt solution and approximately $1 \times 10^6 - 3 \times 10^6$ cells were cultivated in 30 mm plastic petri dishes (Nunc, Roskilde, Denmark). The cells were incubated at 37° for 1–2 h in RPMI-1640 medium supplemented with 2% foetal calf serum (FCS). Non-adhering cells were then removed by repeated rinsing and fresh medium was added. More than 90% of the remaining adherent cells were defined as macrophages by both morphological and phagocytic criteria.

Binding of antigen to macrophage surface

Unstimulated macrophages cultivated in RPMI medium containing 2% FCS were exposed to labelled antigens in concentration of 1, 10 and 15 μg (10^6 c.p.m.)/ml/ 10^6 cells. The exposure to the labelled antigens was carried out either at 37° in the presence of 10^{-1} M sodium azide or at 4° for 1 h. Following incubation with the antigens, the cells were washed and trypsinized for 10 min with 1 ml of trypsin solution 0.2 mg/ml/plate. The radioactivity in the medium was then counted, and the amount of antigen removed by trypsin was defined as surface antigen.

Uptake by macrophages

^{125}I -labelled antigens were added to 10^6 non-stimulated macrophage monolayers at concentrations of 10 μg and 15 μg /ml/plate. After 1 h of incubation at 37°, the cells were washed with PBS, the macrophages were lysed with 1 ml of 1% sodium dodecyl sulphate (SDS) and the cell-associated radioactivity in the lysate was counted.

In some experiments, the uptake of labelled antigens by stimulated macrophages was carried out in the presence of antisera against antigens, homologous and heterologous to the labelled antigens. The antisera were incubated with 1 μg of the labelled antigen for 15 min before their addition to the plates which contained 3×10^6 cells in 1 ml of MEM. The exposure of the cells to the antigen and antisera was for up to 60 min. At different times cells were lysed with 1% SDS and the cell-associated radioactivity in the lysate was counted.

Degradation of antigen by macrophages

Stimulated macrophages (10^5 cells per plate) were incubated for 30 min or 60 min at 37° with the radiolabelled antigens either free or entrapped in liposomes. The cells were then thoroughly rinsed and fresh medium was added. The radioactivity of the cell lysates and in the medium, total and TCA precipitable, was determined at 10 min intervals during an incubation period of 90 min.

RESULTS

Uptake of (T-T-G-G)-A—L and (T-G-T-G)-A—L by peritoneal macrophages

Unstimulated peritoneal cells were taken from C3H.SW mice and cultivated for 2 h at 37° after which the non adherent cells were removed by extensive rinsing. For determination of the amount of antigen uptake, cultures containing 10^6 viable macrophages were incubated with various amounts of radiolabelled (T-T-G-G)-A—L or (T-G-T-G)-A—L for 1 h. The cultures were then washed, and the cell-associated radioactivity was determined after complete lysis by 1% SDS. The results shown in Fig. 1 were obtained following incubation with 10 μg and 15 μg /ml of both antigens. It can be seen that (T-T-G-G)-A—L, the thymus dependent antigen, is

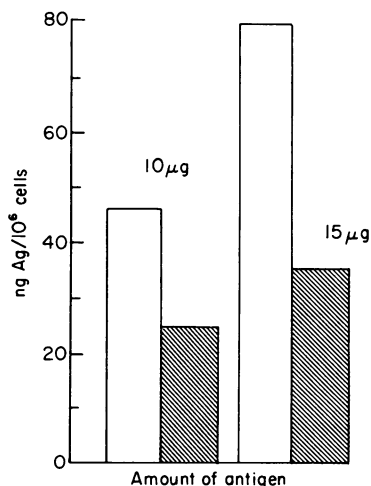


Figure 1. Uptake of radiolabelled antigens by nonstimulated macrophages (10 and 15 μg /ml/ 10^6 cells) following 1 h incubation at 37°. Uptake of [^{125}I]- (T-G-T-G)-A—L (hatched column) and uptake of [^{125}I]- (T-T-G-G)-A—L (open column).

taken by the cells twice as efficient as the thymus independent antigen (T-G-T-G)-A—L. It should be pointed out that these differences were apparent also after eliminating the surface bound antigen by trypsin (results not presented).

In order to increase the extent and rate of antigen's uptake, specific antisera were added to both antigens prior to the incubation with macrophages. The cells used in these experiments were thioglycollate-stimulated peritoneal macrophages. These cells were incubated with the two radiolabelled antigens, (T-T-G-G)-A—L and (T-G-T-G)-A—L (15 μ g) which were preincubated with either anti-(T,G)-A—L or anti-(T-G-T-G)-A—L sera in different dilutions while keeping constant the total amount of mouse sera. Figure 2 demonstrates that anti- (T,G)-A—L sera enhanced the uptake by macrophages of [125 I]- (T-T-G-G)-A—L which is the major determinant of

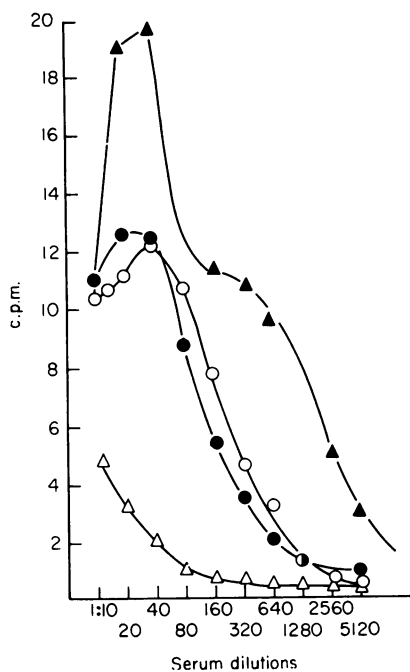


Figure 2. Uptake of labelled antigens by thioglycollate stimulated macrophages in the presence of antisera in different dilutions following 1 h incubation at 37° (●) uptake of [125 I]- (T-T-G-G)-A—L in the presence of C3H.SW anti (T,G)-A—L; (○) uptake of [125 I]- (T-G-T-G)-A—L in the presence of C3H.SW anti (T,G)-A—L; (△) uptake of [125 I]- (T-T-G-G)-A—L in the presence of C3H.SW anti (T-G-T-G)-A—L and (▲) uptake of [125 I]- (T-G-T-G)-A—L in the presence of C3H.SW anti (T-G-T-G)-A—L.

the random (T,G)-A—L. Similarly, the uptake of [125 I]- (T-G-T-G)-A—L by macrophages was increased by anti (T-G-T-G)-A—L sera. Both (T-T-G-G)-A—L and (T-G-T-G)-A—L gave similar patterns of uptake versus antisera dilutions and the minor differences observed can be attributed to differences in either the content of antibodies in the different sera used or in their affinity. Anti-(T,G)-A—L sera were also capable of enhancing the uptake of [125 I]- (T-G-T-G)-A—L by macrophages, whereas anti-(T-G-T-G)-A—L had no effect on the uptake of [125 I]- (T-T-G-G)-A—L (Fig. 2).

Degradation of (T-G-T-G)-A—L and (T-T-G-G)-A—L by macrophages

For the degradation experiments, the two iodinated antigens were entrapped in the inner aqueous volume of liposomes. This approach was chosen in order to by-pass possible differences in uptake and binding of the above antigens. Both antigens (8 or 9 μ g/ml) were entrapped in multilammellar liposomes and the extra-liposomal antigens were removed by gel filtration. The liposomes, containing 300 ng of the respective iodinated antigen ($4-6 \times 10^6$ c.p.m.) were incubated for 45 min with 3×10^6 stimulated macrophages. The excess liposome was then removed by extensive rinsing, and the cultures were supplemented with fresh medium. At different intervals thereafter the cultures were washed and the residual radioactivity in the cells was determined. Assay for the TCA precipitability of the released and of the residual radioactivity indicated that over 80% of the released material was TCA soluble, due to degradation, whereas most of the cell associated radioactivity (60-80%) was TCA insoluble. Figure 3 shows the cell associated TCA precipitable radioactivity. As can be seen both antigens, once taken by the cells, are degraded and released to the medium in a similar manner. In this connection it should be mentioned that the highest value of cell associated radioactivity is not above 80%, which might be due to the fact that technically it was impossible to perform the first measurements at time 0 exactly.

Binding of (T-T-G-G)-A—L and (T-G-T-G)-A—L to the surface of macrophages

Binding of the two antigens to the surface of macrophages was assayed by incubation of the cells with the radioiodinated antigens at 4° or alternatively at 37° in the presence of 10^{-1} M sodium azide.

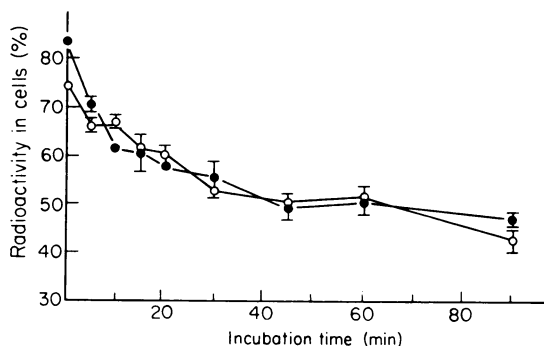


Figure 3. Degradation of radiolabelled antigen by thioglycolate stimulated macrophages following uptake of liposomes in which the antigens were entrapped. Results demonstrate cell associated radio-activity at different time intervals. (O), (T-T-G-G)-A-L; (●), (T-G-T-G)-A-L.

Non stimulated C3H.SW macrophages (10^6 cells per plate) were exposed to different doses of [125 I]- (T-T-G-G)-A-L and [125 I]- (T-G-T-G)-A-L (1, 10 and 15 μ g) for 60 min. The amount of labelled antigen removed by trypsin represented the extra-cellular bound antigen. The results are summarized in Figure 4a and b. As can be seen trypsin treatment, which did not affect the number or viability of the cells, resulted in removal of 82-97% of the antigen after incubation with the cells at 4°, and 72-80% after incubation at 37° in the presence of azide. This

indicates that indeed most of the antigen which was measured in these two systems is bound to the cell membrane. The binding in both systems was concentration-dependent. Comparison between the binding of the two antigens (mainly at 4°) had shown that (T-G-T-G)-A-L, is bound to the macrophage membrane to a larger extent than (T-T-G-G)-A-L, the T dependent polypeptide, almost by a factor of 2. The same pattern of binding was obtained also at 37° in the presence of sodium azide, although the differences between the binding of the two antigens were less pronounced.

DISCUSSION

The two ordered synthetic polypeptides (T-T-G-G)-A-L and (T-G-T-G)-A-L, were found to differ in their need for helper T cells for eliciting efficient immune responses (Schwartz *et al.*, 1976). Furthermore, the immune responses to these antigens were demonstrated to be under different genetic controls (Schwartz *et al.*, 1975).

In the present study we have shown that the two ordered polypeptides differ also in the mode of their interaction with macrophages. The (T-G-T-G)-A-L, which is the thymus independent antigen was bound to macrophage membranes to a higher extent than the T-dependent (T-T-G-G)-A-L (Fig. 4).

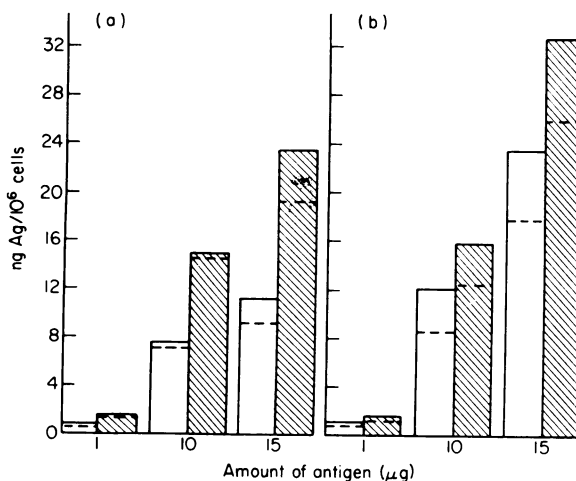


Figure 4. Binding of labelled antigens on macrophage surface, following incubation with 1, 10 and 15 μ g of antigen/ml/ 10^6 cells, (a) at 4°, (b) at 37° in the presence of NaN_3 10^{-1} M. Binding of [125 I]- (T-T-G-G)-A-L (open column) and binding of [125 I]- (T-G-T-G)-A-L (hatched column). The broken lines represent the amount of antigen removed by trypsin.

This has been explored with incubation at 4° and at 37° in the presence of azide. For both conditions most of the labelled antigens could be stripped from the membrane, confirming that we were not measuring uptake. On the other hand, the uptake by macrophages at 37° of (T-G-T-G)-A—L was lower by a factor of two when compared to (T-T-G-G)-A—L. These results indicate the low efficiency of the uptake of (T-G-T-G)-A—L by macrophages taking into account its high binding to membrane.

Uptake was also studied in the presence of antibodies. Antigen-antibody complexes are taken up much more efficiently than free antigen (Fig. 2). As we could not control the concentration and affinity of the specific and the cross-reacting antibody, it would be hard to discuss the quantitative aspects of these data. No major differences were observed in the levels of uptake of the two antigens when (T-T-G-G)-A—L was incubated with anti-(T,G)-A—L and (T-G-T-G)-A—L was reacted with its homologous antiserum. Antiserum to (T-G-T-G)-A—L did not increase the uptake of (T-T-G-G)-A—L but antisera to (T,G)-A—L promoted the uptake of (T-G-T-G)-A—L.

(T-T-G-G)-A—L and (T-G-T-G)-A—L once taken by the macrophages behaved similarly with regard to their susceptibility to degradation as was shown by experiments in which liposomes containing antigens were phagocytosed by the macrophages and the rate of degradation of both antigens was followed (Fig. 3).

The close structural relationships between the two antigens which include an identical size, amino acid composition, extent of branching and optical configuration enable the elucidation of other parameters which might contribute to the T-independency. Both (T-T-G-G)-A—L and (T-G-T-G)-A—L exhibit repeating antigenic determinants, and both are potentially degraded by macrophages to the same extent (Fig. 3), suggesting that each of these two characteristics, or even both of them, are not the direct factor determining the requirement for specific T-cell help. If indeed direct stimulation of B cells is possible when the polymeric antigen is preserved *in vivo* for a long time it could be achieved either with antigens which are unsusceptible to enzymatic degradation as was found for polymers composed of D amino acids (Sela *et al.*, 1972; Bell, 1975) or alternatively when the polymeric antigen is specifically bound to the surface of cells as might be the case with (T-G-T-G)-A—L and as was shown for

KLH. The latter antigen has been reported to be bound to plasma membrane and its immunogenicity was attributed by the authors to the few molecules of antigen which were not degraded (Unanue, Cerottini & Bedford, 1969; Unanue & Cerottini, 1969). Thus, the preferable binding of (T-G-T-G)-A—L to macrophage surface might be related to the direct B cells triggering.

The exact mechanism which determines the efficient binding of (T-G-T-G)-A—L to macrophage surface has not yet been established. However it involves, most probably, both specific features of the macrophage membrane and some unique physico-chemical properties of the antigen as was previously suggested for the KLH antigenic system (Unanue & Cerottini, 1969). The physico-chemical properties which might contribute to the functional behaviour of the two antigens are under investigation at present. It seems plausible that conformational differences, or differences in the tendency to aggregate may influence the binding properties of the two similarly ordered peptide antigens to macrophage surfaces and in turn affect the mode of cell triggering for antibody production.

REFERENCES

- BAR-ELI M. & GALLILY R. (1975) The effect of macrophage hydrolytic enzyme levels on the uptake and degradation of antigen and immune complexes. *J. reticuloendothel. Soc.* **18**, 317.
- BELL G.I. (1975) B lymphocyte activation and lattice formation. *Transpl. Rev.* **23**, 23.
- FELDMANN M. & BASTEN A. (1971) The relationship between antigenic structure and the requirement for thymus derived cells in the immune response. *J. exp. Med.* **134**, 103.
- GREENWOOD F.C., HUNTER W.M. & GLOVER G.S. (1963) The preparation of ¹²⁵I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114.
- MEDLIN J., HUMPHREY J.H. & SELA M. (1970) Studies on synthetic polypeptide antigens derived from multichain polyproline. *Folia biol.* **16**, 156.
- MOZES E., SCHWARTZ, M. & SELA M. (1974) Antibody response of inbred mouse strains to ordered tetrapeptides of tyrosine and glutamic acid attached to multichain polyalanine or polyproline. *J. exp. Med.* **140**, 343.
- SCHWARTZ M., HOOGE R.J., MOZES E. & SELA M. (1976) Role of antigenic structure in cell to cell cooperation. *Proc. nat. Acad. Sci.* **73**, 4184.
- SCHWARTZ M., MOZES E. & SELA M. (1975) The genetic control of the immune response of inbred mouse to conjugates of (Tyr-Tyr-Glu-Glu) and (Tyr-Glu-Tyr-Glu) with multichain poly DL-Alanine as compared with the

- response to the random (T,G)-A—L. *Eur. J. Immunol.* **5**, 866.
- SELA M., FUCHS S. & ARNON R. (1962) Studies on the chemical basis of the antigenicity of proteins. *Biochem. J.* **85**, 223.
- SELA M., MOZES E. & SHEARER J.M. (1972) Thymus independence of slowly metabolized immunogens. *Proc. nat. Acad. Sci. (Wash.)* **69**, 2699.
- SJÖBERG O. & MÖLLER E. (1970) Antigen binding cells in tolerant animals. *Nature (Lond.)*, **228**, 780.
- SMOLARSKY M., TEITELBAUM D., SELA M. & GITLER C. (1977) A simple fluorescent method to determine complement mediated lysosome immune lysis. *J. immunol. Meth.* **15**, 255.
- UNANUE E.R. & CEROTTINI J.-C. (1970) The immunogenicity of antigen bound to the plasma membrane of macrophages. *J. exp. Med.* **131**, 711.
- UNANUE E.R., CEROTTINI, J.-C. & BEDFORD M. (1969) Persistence of antigen on the surface of macrophages. *Nature (Lond.)*, **222**, 1193.