Immunology, 1968, 14, 7.

A Kinetic Study of Antibody Producing Cells in the Spleen of Mice Immunized Intravenously with Sheep Erythrocytes

G. Biozzi, C. Stiffel, D. Mouton, Y. Bouthillier and C. Decreusefond

Institut d'Immuno-Biologie, Hôpital Broussais, 96 rue Didot, Paris 14ème, France

(*Received 6th March* 1967)

Summary. The number of antibody producing cells, i.e. rosette forming cells (RFC) has been studied in the spleen of mice injected intravenously with a full immunizing dose of sheep RBC.

The spleen of a non-immunized mouse contains a background of about 70,000 RFC (normal RFC), which do not appear to be the 'target cells' for antigens of sheep RBC. Our findings suggest that the spleen of a mouse contains about 4000 'target cells' which initiate the immune response to sheep RBC.

In the primary response, the rise of RFC is exponential for about 96 hours with a doubling time of 13 hours involving seven to eight consecutive doubling periods. The peak value of RFC is $1{\cdot}6 \times 10^6$ per spleen.

In the secondary response, the doubling time of RFC is 6–7 hours. The exponential rise lasts 72 hours and includes nine to eleven doubling periods leading to a peak of $3{\cdot}5 \times 10^6$ RFC/spleen.

Adjuvant in the primary response leaves the doubling time of RFC unaltered but prolongs the exponential rise until the 120th hour leading to a peak of $6{\cdot}3 \times 10^6$ RFC/spleen after ten doubling periods. The effects of priming and adjuvant are not fully additive.

INTRODUCTION

Quantitative detection of antibody producing cells in large populations of lymphoid cells isolated from animals immunized with sheep red blood cells (RBC) can be performed by the method of immunocyto-adherence (ICA). ICA is based on the specific fixation of sheep RBC at the surface of the antibody producing cells, i.e. rosette formation (Nota, Liacopoulos-Briot, Stiffel and Biozzi, 1964; Osipova and Karasik, 1964; Zaalberg, 1964; Biozzi, Stiffel, Mouton, Liacopoulos-Briot, Decreusefond and Bouthillier, 1966a). The method of ICA has been applied to the cytodynamic study of the immune response in lymph nodes (Biozzi *et al.*, 1966a) and spleen (Biozzi, Stiffel and Mouton, 1966b; Zaalberg, Van der Meul and Van Twisk, 1966).

Several authors, using the method of localized haemolysis in gel (LHG) (Jerne and Nordin, 1963; Ingraham and Bussard, 1964), have investigated the problem of antibody production at the cellular level (Jerne, Nordin and Henry, 1963; Möller 1965; Šterzl and Riha, 1965; Landy, Sanderson and Jackson, 1965; Jerne, Nordin, Henry, Funi and Koros, 1965 personal communication; Hege and Cole, 1966a; Wortis, Taylor and

Dresser, 1966). A comparison between the two methods has shown, however, that ICA is more sensitive than LHG in detecting antibody producing cells (Biozzi *et al.*, 1966a; Zaalberg *et al.*, 1966), even when the method of LHG has been adapted to reveal the cells producing non-haemolytic antibody by addition of anti-globulin serum (indirect LHG) (Dresser and Wortis, 1965; Šterzl and Riha, 1965). The ICA method, relying on antigen–antibody binding, detects all classes of antibody present at the surface of the producing cells (19S and 7S) (Biozzi *et al.*, 1966b; Cunningham, Smith and Mercer, 1966).

In the present article, the ICA method has been applied to a study of antibody producing cells in the spleen of mice, immunized intravenously with sheep RBC, during both primary and secondary responses as well as under the influence of an adjuvant.

MATERIALS AND METHODS

Adult male Swiss mice of 23–26 g were used.

Immunization

The mice were immunized intravenously with the number of washed sheep RBC indicated in each experiment. The adjuvant used was a heat killed suspension of *Corynebacterium parvum* (*C. parvum**) in saline containing 2·5 mg/ml of bacteria (dry weight). *C. parvum* was administered intravenously at a dose of 0·5 mg/mouse at the times indicated in each experiment.

ICA test

Counting of rosette forming cells (RFC) was performed according to the method previously described (Biozzi *et al.*, 1966a, b), except that phosphate buffered saline (pH 7·2) was used instead of Hanks's solution. The cells giving the ICA form typical rosettes as shown previously. All ICA tests were set up on duplicate samples. The number of RFC was usually determined by observing about 5×10^4 spleen cells. The overall experimental error of the ICA technique is ± 20 per cent.

Serum agglutinins and haemolysins were measured as previously described (Biozzi *et al.*, 1966b).

The ICA test and the serum antibody titration were performed on pooled spleen cells or serum from groups of five mice at different times after immunization. Each point reported in the figures represents the average value from two or three such groups (ten to fifteen mice). A larger number of mice was used to measure the background level of RFC in the spleen before immunization.

RESULTS

In animals immunized intravenously with sheep RBC, the great majority of antibodies are produced by the spleen (Rowley, 1950; Biozzi, Stiffel, Halpern and Mouton, 1960; Landy and Baker, 1966). The dose of antigen used in the present experiments (10^8 sheep RBC) produces a maximum response at both humoral (Dietrich, 1966) and cellular level as measured by LHG (Jerne *et al.*, 1965; Wortis *et al.*, 1966). These findings have been confirmed using the ICA method since the increase in the number of RFC in the spleen and of serum agglutinins are similar after immunization with either 10^8 or 5×10^8 sheep RBC.

* *C. parvum* has been kindly supplied by Professor A. R. Prevot, Institut Pasteur, Paris.

PRIMARY IMMUNIZATION

The immune response as measured by the number of RFC in the spleen and by the titre of serum antibodies in mice immunized with 10^8 sheep RBC is shown in Fig. 1.

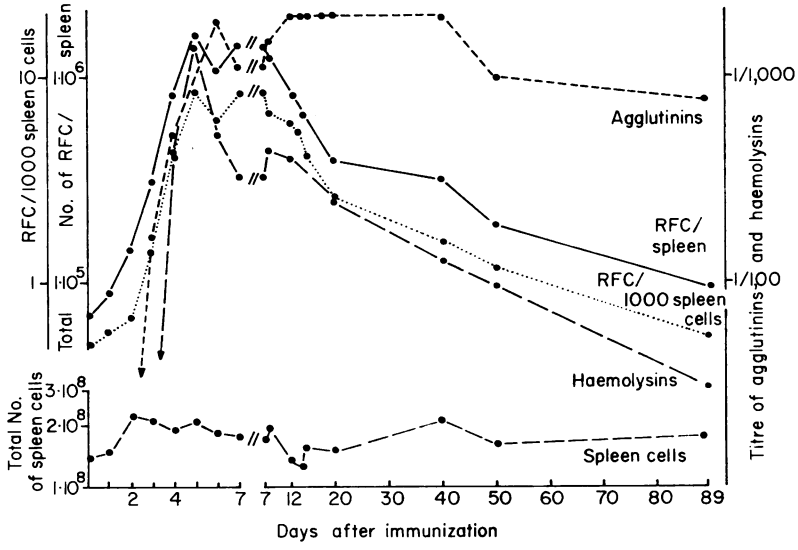


FIG. 1. Primary immunization: cellular and humoral response in mice immunized i.v. with 10^8 sheep RBC.

On the ordinate axis, the value of the normal background level of RFC in the spleen of mice before immunization (about 70,000 RFC/spleen or 0·5/1000 spleen cells) is shown at zero time. The rise in RFC is not quite exponential, since the ascending phase of the curve is somewhat S shaped. The minimum doubling time of RFC calculated on the steepest portion of the curve is about 16 hours. In the descending phase of the curve, two consecutive periods can be distinguished. The first from the 7th to the 20th day has a halving time of 6 days, and the second from the 20th to the 90th day has a halving time of about 35 days.

The level of serum antibody is not appreciably increased until the 3rd day. Thereafter the agglutinins rise rapidly with a doubling time of about 22 hours (calculated between the 3rd and the 6th day). During the ascending phase, there is a close relationship between the rise in RFC and agglutinins. Afterwards the decline of serum agglutinins is slower than that of RFC. The rise of haemolysins is somewhat steeper (doubling time 12 hours) than that of agglutinins followed by a quicker decline. The total number of nucleated cells in the spleen increases markedly soon after antigen injection. The spleen of a normal mouse weighs about 100 mg and yields about $1{\cdot}4 \times 10^8$ nucleated cells ($1{\cdot}4 \times 10^6$/mg). Two days after antigen stimulation the spleen weight is about 160 mg with a cellular yield of $2{\cdot}2 \times 10^8$. This increase persists during the ascending phase of the immune response, thereafter it disappears progressively. It is of interest to note that the peak level of RFC ($1{\cdot}6 \times 10^6$) accounts for about 3 per cent only of the total increase of spleen cells produced by antigen stimulation.

SECONDARY IMMUNIZATION

The secondary response has been studied in animals primed with either 10^8 or 10^5 sheep RBC. A dose of 10^5 sheep RBC does not induce serum antibody but appears to prime.

Secondary response after priming with 10^8 *sheep RBC*

Mice received the second injection of 10^8 sheep RBC 45 days after priming. The resulting secondary response is represented in Fig. 2.

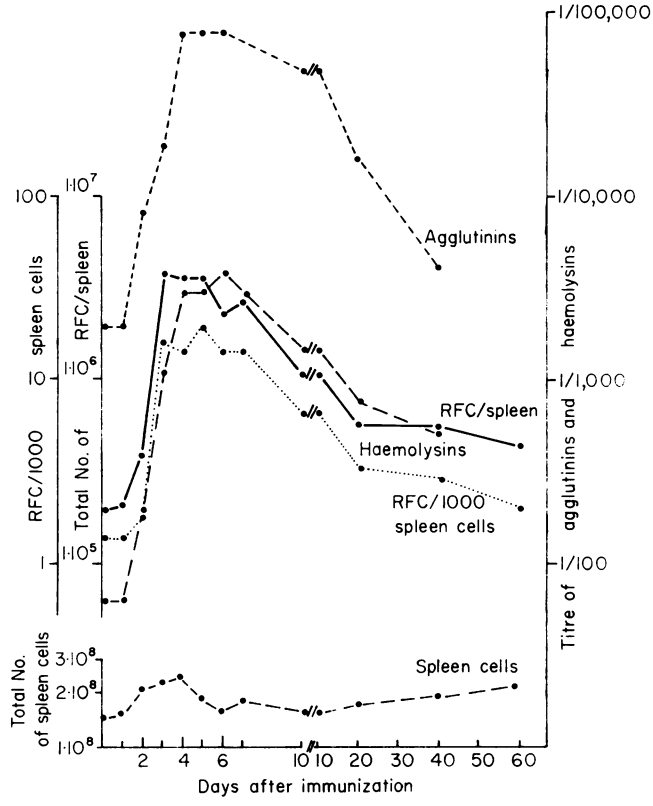


FIG. 2. Secondary immunization: cellular and humoral response in mice primed with 10^8 sheep RBC and challenged i.v. 45 days later with 10^8 sheep RBC.

At the time of the secondary immunization, the cellular and humoral effects of the primary response are still present. The spleen contains about 2×10^5 RFC and the titre of agglutinins is high. Secondary immunization produces a very rapid increase in the number of RFC with a peak level of $3{\cdot}8 \times 10^6$ on the 3rd day. This peak level is twice as high as that of the primary response. The rate of rise of RFC is also greater with a shorter doubling time of about 8 hours. The descending phase comprises an initial rapid decline from the 7th to the 15th day (halving time of 3 days), followed by a slower decrease until the 60th day (halving time 50 days).

The humoral antibody is characterized by a typical secondary response for agglutinins

(doubling time 15 hours), whilst the haemolysin response is similar to the primary one. The total number of spleen cells increases as in the primary response, but the yield of spleen cells/mg is somewhat lower ($1{\cdot}1 \times 10^6$/mg).

Secondary response after priming with 10^5 *sheep RBC*

No modification of the normal background of RFC in the spleen and of serum antibody was detected in groups of mice immunized intravenously with 10^5 sheep RBC and tested 4, 8, 23 and 35 days after immunization. Nevertheless such a dose suffices to develop immunological memory since mice challenged 25 days later with the usual fully immunogenic dose of 10^8 sheep RBC show a typical secondary response at both cellular and humoral level (Fig. 3).

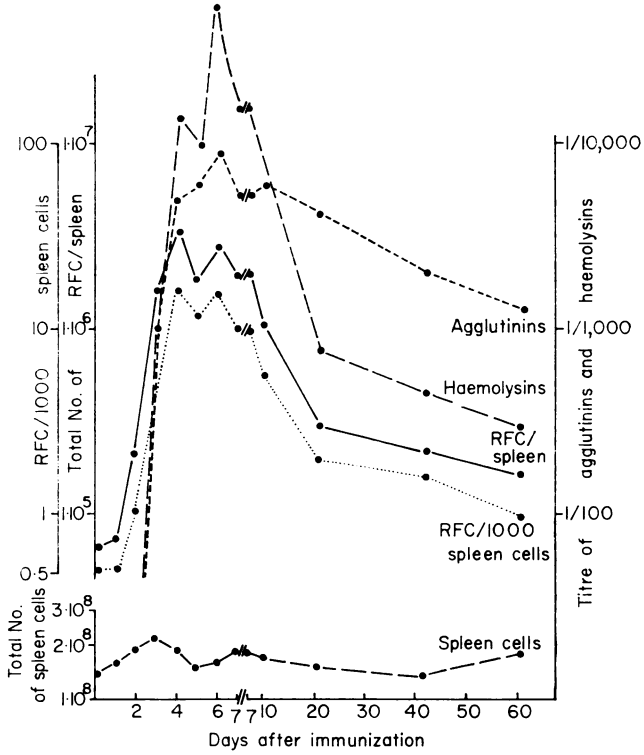


FIG. 3. Secondary immunization: cellular and humoral response in mice primed with 10^5 sheep RBC and challenged i.v. 25 days later with 10^8 sheep RBC.

The RFC rise rapidly to reach a peak of $3{\cdot}5 \times 10^6$ with a doubling time of about 8 hours. The descending phase of RFC is also similar to that observed after the immunogenic priming (Fig. 2). The increased secondary production of both agglutinins and haemolysins is evident and also the substantial increase in the total number of spleen cells.

EFFECT OF AN ADJUVANT ON PRIMARY AND SECONDARY IMMUNIZATION

The adjuvant used was a heat killed suspension of *C. parvum*, which is endowed with a powerful adjuvant effect both on humoral antibody production and on the induction of

delayed hypersensitivity (Neveu, Branellec and Biozzi, 1964). When *C. parvum* is administered intravenously, its adjuvant effect is localized in the spleen which increases markedly in weight and cellularity (Biozzi *et al.*, 1966a). The phagocytic function of the reticuloendothelial cells is also greatly stimulated by *C. parvum* (Halpern, Prevot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreusefond, 1963).

In the experiment represented in Fig. 4, the mice were injected intravenously with 0·5 mg of *C. parvum*; 4 days later they received the usual immunizing dose of 10^8 sheep RBC. The background level of normal RFC in the spleen and the low titre of natural antibodies in the serum are not modified by adjuvant alone, though the total number of spleen cells is already increased at the time of immunization. *C. parvum* increases the primary response about three times since the peak of RFC reaches $6{\cdot}3 \times 10^6$ by the 5th

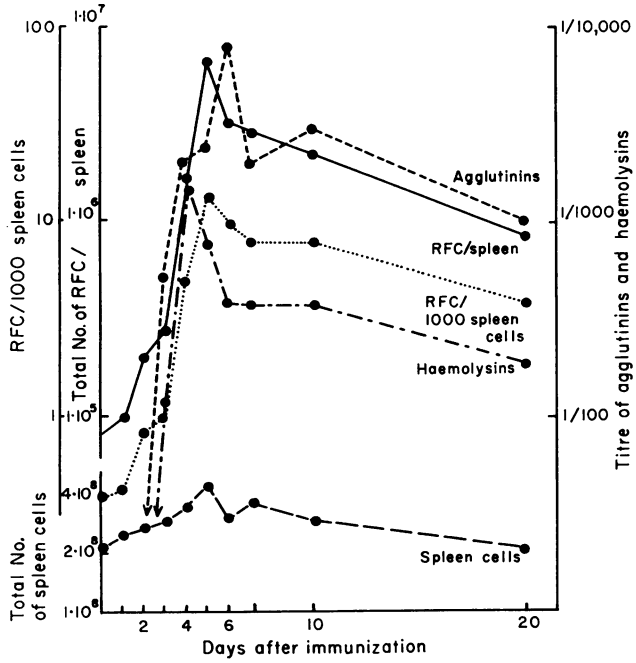


FIG. 4. Effect of adjuvant on the primary immunization: cellular and humoral response in mice injected i.v. with 0·5 mg of *C. parvum* and immunized i.v. 4 days later with 10^8 sheep RBC.

day. The shortest doubling time calculated on the curve is 14 hours. The descending phase was followed only until the 20th day, during which period the halving time of RFC is about 7 days.

The adjuvant *C. parvum* affects only the peak level of agglutinins (doubling time about 19 hours) while haemolysins are not modified. The increase in the total number of spleen cells produced by immunization is evident, though the spleen cell population was already increased by adjuvant at the time of immunization. The composition of the spleen cell population is probably modified by the adjuvant since the yield decreases to $0{\cdot}8 \times 10^6$ cells/mg.

The adjuvant effect of *C. parvum* on the secondary response to sheep RBC is represented in Fig. 5. The mice were primed with 10^5 sheep RBC. After 21 days the animals were

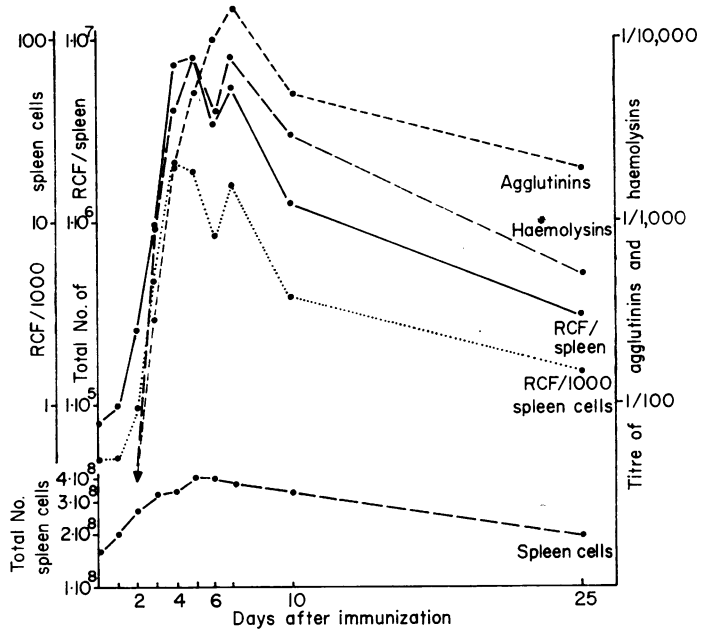


FIG. 5. Effect of adjuvant on the secondary immunization: cellular and humoral response in mice primed with 10^5 sheep RBC and challenged i.v. 25 days later with 10^8 sheep RBC. The adjuvant *C. parvum* was injected i.v. 4 days before the secondary immunization.

re-injected intravenously with 0·5 mg *C. parvum* followed, 4 days later, by the usual immunogenic dose of 10^8 sheep RBC. The addition of priming and adjuvant produces only a small increase in the peak level of RFC ($7 \cdot 6 \times 10^6$) over the effect of adjuvant alone ($6 \cdot 3 \times 10^6$, Fig. 4). The shortest doubling time (10 hours) is intermediary between that of the primary and secondary response. The descending phase of RFC is characterized by a halving time of 2 days between the 5th and the 10th day, similar to that observed after secondary immunization without adjuvant.

DISTRIBUTION OF RFC IN VARIOUS TISSUES AT DIFFERENT TIMES AFTER PRIMARY AND SECONDARY IMMUNIZATION

The number of RFC in various tissues was determined with the same technique used for spleen cells. Blood leucocytes were obtained by mixing equal volumes of heparinized blood with a 4 per cent solution of dextran saline (dextran 500, molecular weight 370,000, Pharmacia Uppsala, Sweden). After standing at 37° for 30 minutes, the leucocyte rich supernatant was separated. The leucocytes were washed twice with buffered saline before performing the ICA test.

Table 1 shows the distribution of RFC in various extra-splenic tissues after primary or secondary immunization. The data were obtained in the same groups of mice used for the experiments represented in Figs. 1 and 2. Clearly, the spleen contains the majority of RFC throughout immunization. A definite increase of RFC is observed in the circulating leucocytes during the early phase of primary and secondary response, corresponding with the peak of RFC in the spleen. These circulating RFC do not seem to settle in other lymphoid tissues, since only occasional small increases of RFC were observed in the lymph nodes and bone marrow.

TABLE 1

DISTRIBUTION OF ROSETTE FORMING CELLS (RFC) AMONG DIFFERENT TISSUES OF MICE IMMUNIZED INTRAVENOUSLY WITH SHEEP ERYTHROCYTES

	Days after immunization	No. of RFC/1000 nucleated cells isolated from: Spleen	Lymph nodes*	Bone marrow	Blood leucocytes†
Primary immunization	5	8·5	0·5	—	1·4
10^8 sheep erythrocytes	12	5	1·2	0·2	0·1
i.v.	20	2·6	1	0·3	0·1
	60	1·2	3	0·1	0·2
	90	0·6	0·8	—	—
	Days after the second injection				
	3	17	1·9	1	9·2
Secondary immunization	4	14·4	0·9	1·2	5
10^8 sheep erythrocytes	5	19	2	—	—
i.v. 45 days apart	7	15	1	—	—
	10	6·7	2·2	—	—
	20	3·3	0·8	—	—
Non-immunized controls	—	0·5	0·8	0·2	0·1

* Total number of lymph node cells isolated: about 3×10^7/mouse.
† Total number of leucocytes isolated: $1{\cdot}5 \times 10^7$/mouse.

ANALYSIS OF THE CYTODYNAMICS OF IMMUNE RESPONSES

A peculiar result given by the ICA method is the surprisingly high level of RFC found in non-immunized animals (normal RFC). It seems very likely that the normal RFC are responsible for the synthesis of natural antibodies against sheep RBC which are present in low concentration in the serum of adult mice (Biozzi *et al.*, 1966b).

It can be argued that not all of the normal RFC are the receptor cells which are stimulated by antigen. Although the background of normal RFC is similar in normal mice (Fig. 1), in mice primed with a low antigen dose (Fig. 3) or treated with an adjuvant (Figs. 4 and 5), the dynamics of the immune responses are different in each case. This lack of relationship suggests that the magnitude of the immune response is independent of the pre-existing level of normal RFC. The experimental results reported in Fig. 6 confirm this view.

This experiment was carried out in adult mice by removing a small fragment of spleen (about 10 mg) under light ether anaesthesia. The number of RFC in the fragment was established. (This value is representative of the content of the normal RFC in the whole spleen since previous experiments had shown that the normal RFC are evenly distributed throughout the spleen.) Two days after partial splenectomy the mice were immunized intravenously with 10^8 sheep RBC. Seven days after immunization, the number of RFC in the spleen and the level of serum agglutinins were determined in each mouse. Single mice present a quite large individual variability in both the number of normal RFC and in the peak level of RFC following immunization; nevertheless these two factors are completely unrelated to each other as shown in Fig. 6. Agglutinin production is also independent of the level of normal RFC. These results indicate that the majority of normal RFC present in the spleen before immunization are not the receptor cells ('target cells') for the antigens of sheep RBC. The term 'target cells' is used without any implication as to whether or not they are precommitted to a given antigen.

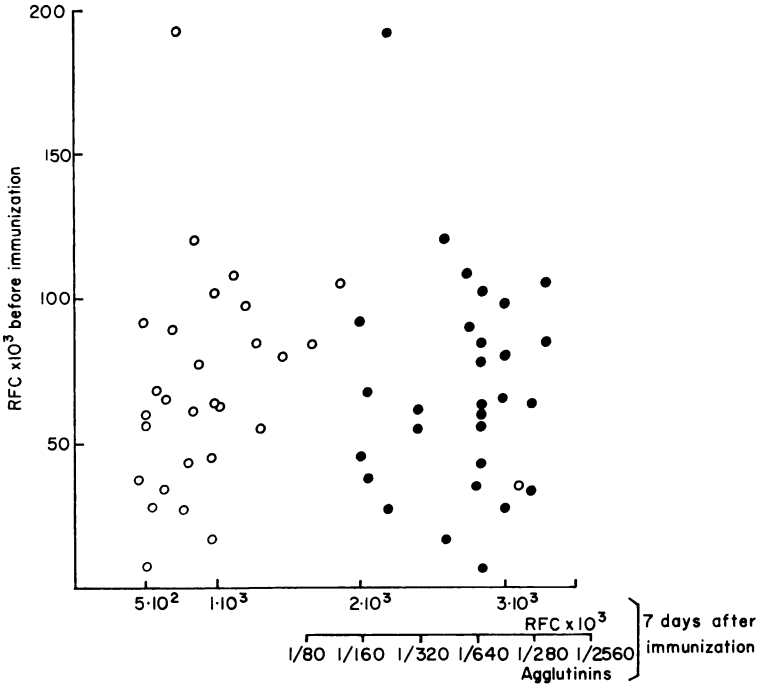
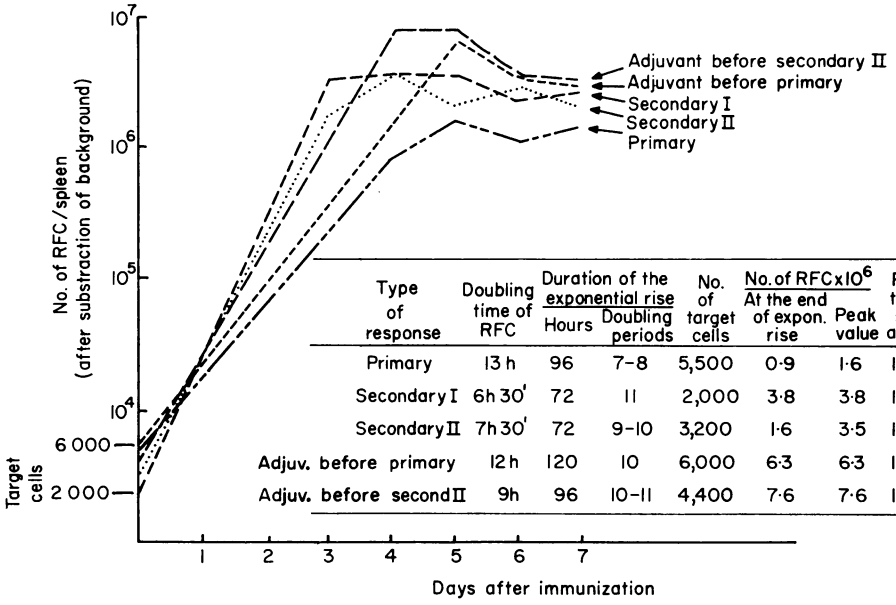


FIG. 6. Lack of relationship between the degree of the immune response (cellular and humoral) and the background level of normal RFC in the spleen. ●, Agglutinin titre; ○, RFC/spleen.



Type of response	Doubling time of RFC	Duration of the exponential rise: Hours	Duration of the exponential rise: Doubling periods	No. of target cells	No. of RFC x10⁶ At the end of expon. rise	No. of RFC x10⁶ Peak value	Peak titre of serum aggutinins
Primary	13 h	96	7-8	5,500	0·9	1·6	1/2,000
Secondary I	6h 30'	72	11	2,000	3·8	3·8	1/78,000
Secondary II	7h 30'	72	9-10	3,200	1·6	3·5	1/10,000
Adjuv. before primary	12 h	120	10	6,000	6·3	6·3	1/10,000
Adjuv. before second II	9h	96	10-11	4,400	7·6	7·6	1/15,000

FIG. 7. Cytodynamics of the different types of immune response after subtraction of the background of normal RFC. Determination of the kinetic constants characteristic for each type of immune response. Extrapolation for initial number of 'target cells'. Secondary I: mice primed with 10^8 sheep RBC; Secondary II: mice primed with 10^5 sheep RBC.

This background of normal RFC interferes with the precise detection of the onset of the immune response, since the initial rise of RFC produced by the immunization is obscured by the number of normal RFC. Consequently the ascending phases of all the curves (Figs. 1–5) are not quite exponential. In Fig. 7 the ascending curves have been corrected by subtracting from each experimental point the values of the background of normal RFC (reported on the ordinate axes at zero time). After correction the rise of RFC follows strictly an exponential function of time for all types of immune responses, during 3–5 days after immunization. The kinetic constants governing each response, therefore, have been calculated (Fig. 7). Assuming a constant rate of increase of RFC from the beginning of the immune response, we have calculated by extrapolation to zero time the number of 'target cells' initially stimulated by sheep RBC. The figures obtained by such an extrapolation fall within the range of 2000 and 6000 for all responses. This variability is certainly not significant so that the number of 'target cells' can be estimated as being roughly 4000/spleen. This level does not appear to be markedly modified by either priming or by adjuvant.

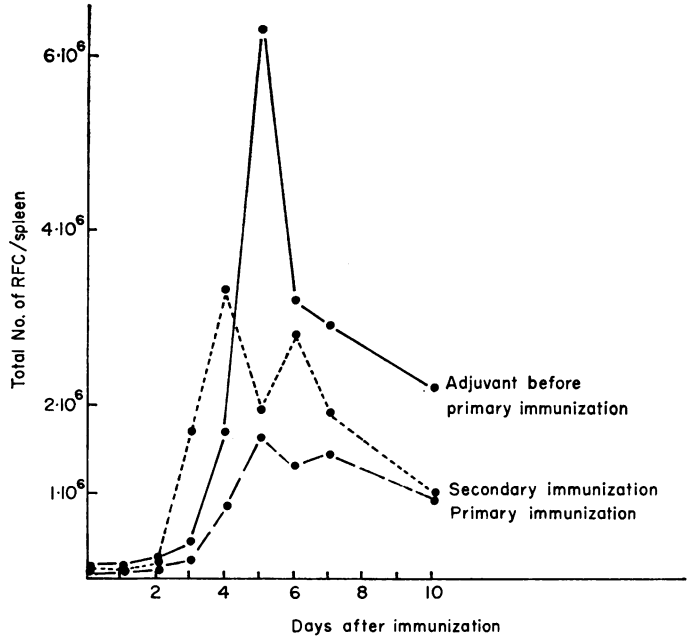


FIG. 8. Cytodynamics of the immune responses represented in numerical co-ordinates (secondary immunization after priming with 10^5 sheep RBC).

LIMITING FACTORS OF THE IMMUNE RESPONSE

A striking feature of all immune responses described above is the sudden change between the phase of rapidly expanding population of RFC and its decline (see Fig. 8), where the data of Figs. 1, 2 and 4 are plotted on a direct numerical scale. It seems probable that some limiting factor suddenly arrests further increase of RFC in the spleen. The experiments reported in Fig. 9 suggest that such a limiting factor may be a shortage of antigen. An additional antigen supply of 5×10^8 sheep RBC was administered intravenously to the mice on the 4th day after primary immunization with 10^8 sheep RBC

(Fig. 9a). The exponential rise was prolonged until the 8th day leading to a peak of $4{\cdot}3 \times 10^6$ RFC. However, other still unknown factors limit the rise of antibody forming cells even in the presence of antigen excess, since a second antigen supply (5×10^8 sheep RBC) given on the 8th day does not prolong the increase in RFC (Fig. 9a).

Similar results were obtained in mice undergoing a primary response after the administration of *C. parvum* (Fig. 9b). The animals were immunized as in the experiment reported in Fig. 4. Five times 10^8 sheep RBC administered intravenously on the 4th day prolonged the exponential rise of RFC for 3 days.

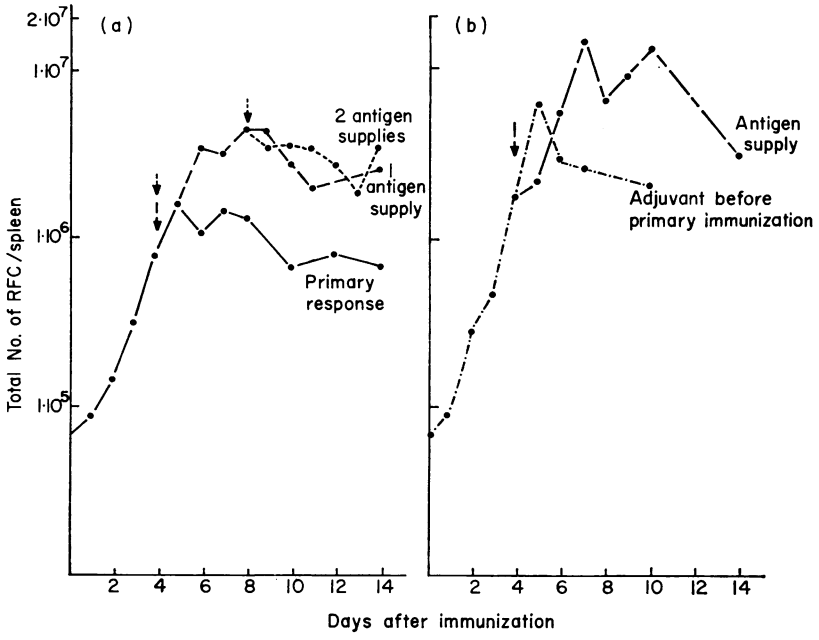


FIG. 9. Effect of additional antigen supplies (5×10^8 sheep RBC) administered i.v. during the ascending phase of the immune response. (a) Primary immunization; (b) primary immunization in mice treated with adjuvant (0·5 mg of *C. parvum* i.v. 2 days before immunization). The arrows indicate the time of antigen supply.

DISCUSSION

It is well known that chiefly 19S immunoglobulins are produced at the beginning of immunization, while 7S antibodies dominate in the later phases or during secondary immunization. The parallel between RFC and agglutinins during the primary response is broken after the 10th day of immunization, since the number of RFC falls while high agglutinin levels persist (Fig. 1). The 19S immunoglobulins have a shorter circulatory half life (0·5 days) than 7S globulins (2·5–5·4 days, Fahey and Sell, 1965). Therefore, a smaller number of antibody producing cells will be required to sustain a high level of the long living antibody prevailing in the advanced phase of primary response. Once more possible extrasplenic localization of antibody producing cells may contribute to this discrepancy. During the secondary response (Figs. 2 and 3) and after adjuvant (Figs. 4 and 5), presumably 7S antibody production dominates and the parallel between RFC and agglutinins persists throughout the immune response.

The shortest doubling times of RFC calculated on the steeper rise of the uncorrected curves (Figs. 1–5) is somewhat longer than that resulting from the corrected curves by subtracting the background of normal RFC (Fig. 7). This correction seems to be justified by the lack of relationship between the level of the normal RFC and the degree of the immune response (Fig. 6). Such a negative relationship has been observed by various investigators using the LHG method (Šterzl, Vesely, Jilek and Mandel, 1965b; Hege and Cole, 1966b; Wigzell, 1966). The rise of RFC after correction follows a strict exponential function of time throughout the entire ascending phase of all types of immune responses studied (Fig. 7). Therefore, the kinetic constants calculated from Fig. 7 should be considered more precise than those resulting from the non-corrected curves.

The doubling time of RFC during the primary response (13 hours) is longer than that of 7–9 hours reported by various authors using the LHG method which detects only haemolytic (19S) antibody (Jerne *et al.*, 1963; Koros, Fuji and Jerne, 1966; Hege and Cole, 1966a). This difference suggests a shorter doubling time of 19S forming cells compared to the whole population of antibody producing cells detected by the ICA method. The doubling times of RFC obtained in the present study (Fig. 7) are somewhat longer than those of 9 hours reported by Albright and Makinodan (1965) for blast cells involved in antibody production in both primary and secondary responses. A longer doubling time (12 hours) for the secondary response has been obtained by other investigators (Schooley, 1961; Nossal and Mäkela, 1962). The exponential rise of RFC in the secondary response encompasses nine to eleven doubling periods. This finding is in good agreement with the results obtained by the above investigators (also Mäkela and Nossal, 1962 and Makinodan and Albright, 1967).

The extrapolation of the number of 'target cells' in Fig. 7 deserves comment. It is based upon the assumption that the rise of RFC occurs at a constant rate starting immediately after antigen stimulation without a latent phase. The existence of a latent phase will obviously lead to an under-estimate of the number of 'target cells'. Such a latent phase is not generally admitted, but should be of only short duration (Uhr and Finkelstein, 1967). Allowing a maximum latent period of 12 hours, the level of 'target cells' would rise to about 8000. As the latent period is probably shorter than 12 hours, it will interfere little with the extrapolation shown in Fig. 7

Since the dose of sheep RBC used is a maximal one, it is likely that all the 'target cells' in the spleen are stimulated by the antigen. The whole spleen of a mouse would therefore contain about 4000 cells which are capable of responding immunologically against the antigens of sheep RBC. Makinodan and Albright (1963), and Vasquez and Makinodan (1966) using the *in vivo* culture technique estimated that the normal mouse spleen contains thirty to 150 'target cells', this number being increased about 100-fold after priming. Jerne *et al.* (1963) using the LHG technique arrived at a similar conclusion. On the other hand, Playfair, Papermaster and Cole (1965) and Kennedy, Till, Siminovitch and McCulloch (1966) using the method of haemolytic focus assay estimated that the mouse spleen contains 1000–5000 cells sensitive to the antigens of sheep RBC. With a different technique based on radiation sensitivity of antibody producing cells, Kennedy, Till, Siminovitch and McCulloch (1965) arrived at the conclusion that a mouse spleen should contain at least 1000 antigen sensitive cells. Our data are in agreement with these findings.

The experimental data presented here are compatible with the following cytodynamic model: starting from a constant level of 'target cells' (about 4000) each type of immune response is governed by only two kinetic constants: (1) The doubling time of the antibody

forming cells during the exponential rise. (2) The number of doubling periods encompassed in the exponential rise. A considerable mass of experimental findings indicates that cellular multiplication is a dominant phenomenon of the immune response. The results of Figs. 1–5 show a rapid increase in the total number of spleen cells after antigen stimulation. This phenomenon is probably due to cell multiplication. The number of RFC represents only about 3 per cent of the overall increase in the total number of spleen cells for each type of response studied. Therefore the process of cellular multiplication is not restricted to the group of cells actually producing antibody. A similar result has been reported by Wortis *et al.* (1966).

The importance of the amount of the antigen on the duration of the cellular immune response appears clearly from the experiment represented in Fig. 9. A similar effect on the production of serum antibodies has been observed by Uhr (1964) and by Svehag and Mandel (1964).

REFERENCES

ALBRIGHT, J. F. and MAKINODAN, T. (1965). 'Dynamics of expression of competence of antibody producing cells.' *Molecular and Cellular Basis of Antibody Formation* (Ed. by J. Šterzl), p. 427. Czechoslovakian Academy of Science, Prague.

BIOZZI, G., STIFFEL, C., HALPERN, B. N. and MOUTON, D. (1960). 'Recherches sur le mécanisme de l'Immunité non spécifique produite par les Mycobactéries.' *Rev. franç. Étud. clin. biol.*, **5**, 876.

BIOZZI, G., STIFFEL, C., MOUTON, D., LIACOPOULOS-BRIOT, M., DECREUSEFOND, C. and BOUTHILLIER, Y. (1966a). 'Etude du phénomène de l'Immunocytoadherence au cours de l'immunisation.' *Ann. Inst. Pasteur*, **110**, (suppl. 3), 7.

BIOZZI, G., STIFFEL, C. and MOUTON, D. (1966b). 'A study of the antibody containing cells in the course of immunization.' '*Intern. Symp. on Immunity, Cancer and Chemotherapy*', *Buffalo* 1966 (Ed. by E. Mihich). Academic Press, New York.

CUNNINGHAM, A. J., SMITH, J. B. and MERCER, E. H. (1966). 'Antibody formation by single cells from lymph nodes and efferent lymph of sheep.' *J. exp. Med.*, **124**, 701.

DIETRICH, F. M. (1966). 'The immune response to heterologous red cells in mice.' *Immunology*, **10**, 365.

DRESSER, D. W. and WORTIS, H. H. (1965). 'Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency.' *Nature (Lond.)*, **208**, 859.

FAHEY, J. L. and SELL, S. (1965). 'The immunoglobulins of mice. V. The metabolic (catabolic) properties of five immunoglobulin classes.' *J. exp. Med.*, **122**, 41.

HALPERN, B. N., PREVOT, A. R., BIOZZI, G., STIFFEL, C., MOUTON, D., MORARD, J-C., BOUTHILLIER, Y. and DECREUSEFOND, C. (1963). 'Stimulation de l'activité phagocytaire du système réticulo-endothélial provoquée par *Corynebacterium parvum*.' *J. Reticuloendothelial Soc.*, **1**, 77.

HEGE, J. S. and COLE, L. J. (1966a). 'Antibody plaque-forming cells: kinetics of primary and secondary responses.' *J. Immunol.*, **96**, 559.

HEGE, J. S. and COLE, L. J. (1966b). 'Antibody plaque-forming cells in non sensitized mice: effect of neonatal thymectomy, phytohemagglutinin and X-radiation.' *Fed. Proc.*, **25**, 305.

INGRAHAM, J. and BUSSARD, A. (1964). 'Application of localized hemolysin reaction for specific detection of individual antibody forming cells.' *J. exp. Med.*, **119**, 667.

JERNE, N. K. and NORDIN, A. A. (1963). 'Plaque formation in agar by single antibody producing cells.' *Science*, **140**, 405.

JERNE, N. K., NORDIN, A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody producing cells.' *Cell-Bound Antibodies* (Ed. by B. Amos and H. Koprowski), p. 109. The Wistar Institute Press, Philadelphia.

KENNEDY, J. C., TILL, J. E., SIMINOVITCH, L. and MCCULLOCH, E. A. (1965). 'Radiosensitivity of the immune response to sheep red cells in the mouse as measured by the hemolytic plaque method.' *J. Immunol.*, **94**, 715.

KENNEDY, J. C., TILL, J. E., SIMINOVITCH, L. and MCCULLOCH, E. A. (1966). 'The proliferative capacity of antigen-sensitive precursors of hemolytic plaque-forming cells.' *J. Immunol.*, **96**, 973.

KOROS, A. M. C., FUJI, H. and JERNE, N. K. (1966). 'Kinetics of proliferation of clones of antibody producing cells.' *Fed. Proc.*, **25**, 305.

LANDY, M., SANDERSON, R. P. and JACKSON, A. L. (1965). 'Humoral and cellular aspects of the immune response to the somatic antigen of *Salmonella enteritidis*.' *J. exp. Med.*, **122**, 483.

LANDY, M. and BAKER, P. J. (1966). 'Cytodynamics of the distinctive immune response produced in regional lymph nodes by *Salmonella* somatic polysaccharide.' *J. Immunol.*, **97**, 670.

MÄKELA, O. and NOSSAL, G. J. V. (1962). 'Autoradiographic studies on the immune response. II. DNA synthesis amongst single antibody producing cells.' *J. exp. Med.*, **115**, 231.

MAKINODAN, T. and ALBRIGHT, J. F. (1963). 'Cytokinetics of antibody response.' *Immunopathology, 3rd Int. Symp.* (Ed. by P. Grabar and P. Miescher), p. 99. Schwabe, Basel.

MAKINODAN, T. and ALBRIGHT, J. F. (1967). 'Proliferative and differentiative manifestations of cellular immune potential.' *Progr. Allergy*, **10**, 1.

MOLLER, G. (1965). '19S antibody production against soluble lipopolysaccharide antigens by individual lymphoid cells "in vitro".' *Nature (Lond.)*, **207**, 1166.

NEVEU, T., BRANELLEC, A. and BIOZZI, G. (1964). 'Propriétés adjuvantes de Corynebacterium parvum sur la production d'anticorps et sur l'induction de l'hypersensibilité retardée envers des protéines conjuguées.' *Ann. Inst. Pasteur*, **106**, 771.

NOSSAL, G. J. V. and MÄKELA, O. (1962). 'Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation.' *J. exp. Med.*, **115**, 209.

NOTA, N. R., LIACOPOULOS-BRIOT, M., STIFFEL, C. and BIOZZI, G. (1964). 'L'Immunocyto-adherence: une méthode simple et quantitative pour l'étude "in vitro" des cellules productices d'anticorps.' *C. R. Acad. Sci. (Paris)*, **259**, 1277.

OSIPOVA, P. V. and KARASIK, O. A. (1964). 'Specificity of erythrocytes adhesion to the lymph cell surface "in vitro".' *Bull. exp. biol. Med.*, **58**, 100.

PLAYFAIR, J. H., PAPERMASTER, B. W. and COLE, L. J. (1965). 'Focal antibody production by transferred spleen cells in irradiated mice.' *Science*, **149**, 998.

ROWLEY, D. A. (1950). 'The effect of splenectomy on the formation of circulating antibody in the adult male albino rat.' *J. Immunol.*, **64**, 289.

SCHOOLEY, J. C. (1961). 'Autoradiographic observations of plasma cell formation.' *J. Immunol.*, **86**, 331.

ŠTERZL, J. and RIHA, I. (1965). 'Detection of cells producing 7S antibodies by the plaque technique.' *Nature (Lond.)*, **208**, 858.

ŠTERZL, J., VESELY, J., JILEK, M. and MANDEL, L. (1965b). 'The inductive phase of antibody formation studied with isolated cells.' *Molecular and Cellular Basis of Antibody Formation*' (Ed. by J. Šterzl), p. 463. Czechoslovakian Academy of Sciences, Prague.

SVEHAG, S. E. and MANDEL, B. (1964). 'The formation and properties of poliovirus neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis and sensitivity to X-irradiation.' *J. exp. Med.*, **119**, 21.

UHR, J. W. (1964). 'The heterogeneity of the immune response. The quantity and nature of antigen can regulate a variety of immunological functions.' *Science*, **145**, 457.

UHR, J. W. and FINKELSTEIN, M. S. (1967). 'The kinetics of antibody formation.' *Progr. Allergy*, **10**, 37.

VAZQUEZ, J. J. and MAKINODAN, T. (1966). 'Cytokinetics events following antigenic stimulation.' *Fed. Proc.*, **35**, 1727.

WIGZELL, H. (1966). 'Antibody synthesis at the cellular level: some studies on natural anti-sheep red cell antibodies in the mouse.' *J. Immunol.*, **97**, 608.

WORTIS, H. H., TAYLOR, R. B. and DRESSER, D. W. (1966). 'Antibody production studied by means of the LHG assay. I. The splenic response of CBA mice to sheep erythrocytes.' *Immunology*, **11**, 603.

ZAALBERG, O. B. (1964). 'A simple method for detecting single antibody forming cells.' *Nature (Lond.)*, **202**, 1231.

ZAALBERG, O. B., VAN DER MEUL, V. A. and VAN TWISK, J. M. (1966). 'Antibody production by single spleen cells: a comparative study of cluster and agar-plaque formation.' *Nature (Lond.)*, **210**, 544.