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Correlations between immunoglobulin- and antibody-synthesizing cells during primary and secondary immune responses of rats immunized with peroxidase

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Summary. The development of cells synthesizing immunoglobulins without detectable antibody activity and of antibody-synthesizing cells was studied during primary and secondary immune responses of rats immunized with horseradish peroxidase.

After primary immunization with peroxidase emulsified in Freund's complete or incomplete adjuvant, the first antibody-producing cells appeared 4 days after injection. They were preceded by cells synthesizing IgG and IgM without antibody function, appearing 3 days after giving antigen. The ratio between the latter and the former population of cells regularly decreased during the primary response. Seventy to 100 per cent of cells synthesizing immunoglobulins without antibody activity were induced by the antigen, the remainder being induced by the adjuvant. In both populations, the positive cells were always immature or mature plasmocytes.

At various times after primary injection, animals received a booster inoculation of soluble peroxidase or of peroxidase emulsified in Freund's adjuvant. Antibody-producing cells, in early stages of differentiation, appeared between 2 and 3 days after challenge and were not preceded by cells synthesizing immunoglobulins without antibody function. These latter cells were reduced or absent after secondary challenge. Increasing the sensitivity of detection of active sites of antibodies, by using indirect methods of staining with fixed or unfixed cells gave no increase of antibody-producing cells.

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INTRODUCTION

It is well known that antigen induces the appearance of specific antibodies directed against the molecule injected as well as immunoglobulins without detectable antibody function which have been called 'non-specific immunoglobulins' (Boyd and Bernard, 1937; Pappenheimer, Lundgren and Williams, 1940; Askonas and Humphrey, 1958; Helmreich, Kern and Eisen, 1961; Humphrey, 1963; Drizlikh, 1965; Gurvitch and Drizlikh, 1967; Gurvitch and Nikolaeva, 1971). Recently, it has been shown that antibodies and immunoglobulins without antibody activity were synthesized by different populations of cells (Urbain-Vansanten, 1970; Antoine and Avrameas, 1973; Moticka, 1974; Miller, Ternynck and Avrameas, 1974, 1975). Furthermore, an increase in intracellular immunoglobulins in practically all splenic lymphoid cells was found to occur after primary immunization with sheep red blood cells (Packmann, Killander and Wigzell, 1974). It is not known if the population of cells synthesizing immunoglobulins without detectable antibody function is due to direct stimulation by the antigen or indirect stimulation due to the release of mitogenic or stimulating factors from lymphoid cells (Rubin and Coons, 1971).

Investigators have attempted to find relationships existing between antibody- and immunoglobulin-producing cells. De Vos-Cloetens, Minsart-Baleriaux and Urbain-Vansanten (1971) showed that tobacco mosaic virus and bovine serum albumin induced the formation of antibodies and non-specific immunoglobulins when injected independently, but when these antigens were injected simultaneously the number of immunoglobulin-producing cells was approximately equal to the sum of immunoglobulin-synthesizing cells which appeared after separate inoculation of the two antigens. Specific anti-ovalbumin antibodies and immunoglobulins without detectable antibody function, both appearing in the blood of rabbits after ovalbumin administration, were found to possess common idiotypic specificities (Oudin and Cazenave, 1971). A similar observation was made at the cellular level with cells synthesizing immunoglobulins without antibody activity and possessing common idiotypic specificities with cells synthesizing antibody directed against the injected antigen (peroxidase or ovalbumin) (Cazenave, Ternynck and Avrameas, 1974). Furthermore it was shown that, compared to the immunoglobulin-producing cells, the doubling time of the antibody-synthesizing cells was shorter (Miller *et al.*, 1974, 1975). Here, it was also found that some cells contained antibody confined to the Golgi complex and some ergastoplasmic cisternae, while other areas, free of detectable antibody activity, could be stained with enzyme-labelled anti-Ig antibody.

All these results indicate that there is a relationship between antigen and immunoglobulin without antibody activity. It is not clear whether in these different studies non-specific immunoglobulin production was stimulated by similar mechanisms. For instance, the increase of intracellular immunoglobulins in all lymphoid cells observed by Packmann *et al.* (1974) might have been due to the action of mitogenic or stimulating factors appearing after antigenic stimulation. Furthermore it is evident that the method employed to detect the antibody-synthesizing cells, the antigen used and its route of administration, and the time after antigen administration the observations are made can also enormously influence the results obtained. In the present paper we have studied the kinetics of proliferation of Ig-synthesizing and antibody-synthesizing cells during the primary and the secondary response to horseradish peroxidase. The sequential appearance of the two populations during the primary response, the number of cells present in these two populations, and the absence or great decrease of Ig-producing cells during the secondary response support our previously formulated hypothesis, i.e. recruitment of antibody-producing cells from Ig-containing cells.

MATERIALS AND METHODS

Animals

Five-month-old Sprague–Dawley, W.A.G. or oFA rats of both sexes were used. Similar results were obtained with all these strains.

Immunizations

Immunization was carried out by injecting subcutaneously in both hind footpads 1 mg of antigen emulsified in Freund's complete or incomplete adjuvant (FCA or FIA) (Difco Laboratories, Detroit, Michigan). Animals received for the secondary response at various times after priming and by the same route 0·5 mg or 1 mg of antigen given as a solution in phosphate-buffered saline (10^{-2} M phosphate buffer, pH 7·4, 0·15 M NaCl) or emulsified in FIA.

Antigen

Peroxidase (Po) RZ = 3 was purchased from Boerhinger, Mannheim, Germany. Purity of the preparations employed for immunizations was checked by double immunodiffusion and immunoelectrophoresis. Antisera obtained in hyperimmunized sheep or oFA rats revealed only a single precipitation line.

Preparations of cells

At least three rats immunized with Po and three control rats were studied for each day examined. At various times after primary or secondary challenge, animals were killed and their popliteal lymph nodes removed and placed in cold Hanks's medium (Institut Pasteur, France) containing 10 mM Hepes (Sigma Chemical Company, St Louis, Missouri). Lymph nodes were teased with fine forceps and cells were washed three times by successive centrifugations as described previously (Gonatas, Antoine, Stieber and Avrameas, 1972). Cells were counted, and viability was tested by trypan blue exclusion. In all experiments, about 70 per cent cell viability was found. 0·2 ml of cellular suspension containing 5×10^5 cells/ml and 5 mg/ml of BSA or hen ovalbumin (Sigma Chemical Company, St Louis, Missouri) was centrifuged with a Shandon cytocentrifuge

(Shandon Southern Instruments Limited, England) for 7 min at 1000 rev/min. Cells were spread on a small round area of about 6·2 mm diameter. Slides were left overnight, then fixed for 15 min in 4 per cent paraformaldehyde in 0·2 M cacodylate buffer, pH 7·4, and washed three times with PBS. The slides were then immediately incubated with antigen or labelled antibodies.

Preparation of antisera and of purified antibodies

Rabbit anti-rat Ig antisera were prepared as described earlier (Gonatas *et al.*, 1972). Purified rabbit antibodies directed against rat Fab and rat Fcγ fragments were prepared following procedures already described (Antoine and Avrameas, 1973, 1974). Anti-Fcγ reacted with IgG2a, IgG2b and slightly with IgG1. Purified antibodies to anti-rat μ chain were prepared according to the following procedures. Rabbits were injected with rat IgM IR202 myeloma protein. Antiserum was absorbed once on a rat pseudoglobulin immunoadsorbent prepared with glutaraldehyde (Avrameas and Ternynck, 1969). Pseudoglobulins contain little IgM and permit the elimination of antibodies directed against non-IgM components. An additional adsorption was performed on a rat IgG immunoadsorbent to remove the remaining anti-γ chain and anti-light chain antibodies. Anti-μ chain antibodies were isolated on IR202 euglobulins precipitated from sera of rats bearing IR202 myeloma, filtered on Sephadex G-200 (Pharmacia, Uppsala, Sweden) and polymerized with glutaraldehyde (Bazin, Beckers and Querinjean, 1974). These antibodies gave a strong precipitin reaction with IgM and reacted slightly with a non-immunoglobulin α component. Sheep anti-rabbit Ig antibodies, free of cross-reactivity with rat Ig, were prepared by adsorbing sheep antisera or purified antibodies on a rat IgG immunoadsorbent until the cross-reaction with this antigen disappeared. Some rabbit anti-rat μ chain sera were gifts from Dr R. Oriol (Hôpital Broussais, Paris, France) and from Dr H. Bazin (Department of Experimental Medicine, Bruxelles, Belgium). Rat IgM IR202 myeloma and sera of rats bearing IR202 myeloma were gifts from Dr H. Bazin.

Labelling of purified antibodies

Purified antibodies were labelled with peroxidase following a two-step procedure (Avrameas and Ternynck, 1971) or with alkaline phosphatase from *E. coli* (Worthington Biochemical Corporation, Freehold, New Jersey) (20 u/mg) following a one-step procedure (Avrameas, 1969a).

Detection of immunoglobulin- and antibody-synthesizing cells

To detect cells synthesizing antibodies against peroxidase, the cell suspensions were incubated for 1 h with a 100 μg/ml solution of Po. Po activity was detected according to the method of Graham and Karnovsky (1966). To detect cells synthesizing immunoglobulins, the preparations were incubated for 2–3h with a 125–250 μg/ml solution of anti-rat Fab antibodies labelled with either peroxidase or alkaline phosphatase (AP). Alkaline-phosphatase activity was detected using Fast Red TR Salt and naphthol As Mx Phosphate (Sigma Chemical Company, St Louis, Missouri) as substrate (Burstone, 1962; Avrameas, Taudou and Ternynck, 1971). IgG- and IgM-synthesizing cells were detected by incubating cellular preparations for 3 h with AP-labelled anti-Fcγ and anti-μ antibodies respectively. An indirect method was also used for the detection of IgM-producing cells. Cells were first incubated for 1h with anti-μ antisera and then after washing, for 3 h with sheep anti-rabbit Ig antibodies labelled with AP and which had been previously adsorbed on insoluble rat IgG. The detailed procedures employed have been published elsewhere (Avrameas *et al.*, 1971; Antoine *et al.*, 1973).

After staining, the total number of positive cells present on each slide was counted. The total number of cells was calculated by counting cells present in thirty microscopic fields (objective $\times 40$) and by multiplying the number obtained by the ratio between the total area occupied by the cells and the thirty microscopic fields.

Control experiments

(a) *Immunocytochemical staining.* Control slides incubated with normal IgG labelled with AP or Po were negative except for some cells such as eosinophil polymorphonuclears which have endogenous AP and Po activity. Controls to assess the specificity of the antibody staining were made by incubating slides first in PBS and then in the Po substrate. Only some eosinophils and macrophages containing endogenous peroxidase activity showed a positive reaction.

(b) *Specificity of antibodies.* Controls to check the specificity of the monospecific antibodies employed

were performed by first mixing the labelled antibodies with the homologous antigen preparation and then performing the staining. Staining with anti-Fab was inhibited by incubating conjugate for 1 h at 37° with IgG or IgM solution. Staining with anti-Fcγ was inhibited by previous incubation of the conjugate with IgG solution but not with IgM. Conversely, staining of cells with anti-IgM conjugate was specifically inhibited with IgM solution but not with IgG solution.

(c) *Sensitivity of the methods.* It was considered that the two methods used to detect the active sites of antibodies and the antigenic determinants of the immunoglobulins were not equally sensitive. Attempts were made to increase the sensitivity of the detection of antibodies: (1) a two-step procedure in which the cells were incubated with Po (or PBS for the control), then washed and incubated with sheep anti-Po antibody labelled with AP; (2) a three-step procedure in which the cells were successively incubated in: (a) Po solution; (b) rat anti-Po antiserum; (c) Po solution. Details have been described elsewhere (Avrameas, 1969b). The following results were obtained: the two-step procedure was less sensitive than the one-step incubation. Often the percentage of antibody-containing cells was lower with the two-step method, which only allowed the detection of cells with a heavy staining distributed throughout the cytoplasm; cells where staining was localized to a small area of the cytoplasm were hardly detected because of the background obtained with this method. With the three-step procedure we always found about twice the number of positive cells detected with Po alone. However, most of the additional cells detected by this indirect method were very slightly stained and were also detected if anti-Po antiserum was replaced by normal serum. It was therefore concluded that this slight staining represented a low background due to the long incubation of slides with Po and serum.

Attempts were made to increase the detection of antibody-producing cells using cytocentrifuged and dried, unfixed cells. It was considered possible that the fixation used (4 per cent paraformaldehyde in 0·2 M cacodylate buffer, pH 7·4) altered the antibody-active sites more than the antigenic determinants; this differential alteration could be explained by the relative concentration of these determinants. Approximately the same percentage of antibody-producing cells was obtained with fixed and unfixed cellular preparations. Similar results for Ig-synthesizing cells were obtained with fixed and unfixed cellular preparations. Immunohistochemical staining was intense with unfixed cells, indicating a good penetration of the conjugates.

RESULTS

Primary response

Kinetics of proliferation of antibody- and immunoglobulin-producing cells

After a single injection of peroxidase in complete or incomplete Freund's adjuvant, the size of the popliteal lymph node and the number of lymphoid cells contained in it, increased rapidly to their maximum 11–15 days after stimulation and then diminished slightly. Even 13 months after primary injection the nodes were still about five times bigger than normal nodes. Rare antibody-producing cells appeared for the first time, 4 days after injection, and were always present for the 13-month period of the experiment (Fig. 1). A few positive cells were observed during the first 10 days, then they increased

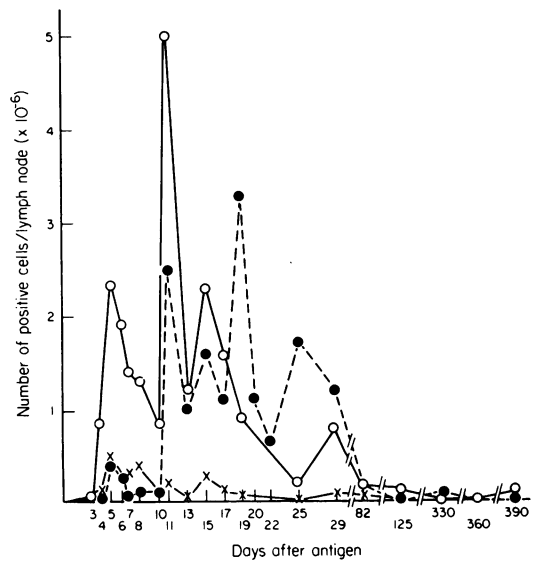

Figure 1. Kinetics of proliferation of antibody-synthesizing cells (● - - - ●) and of cells synthesizing immunoglobulins without detectable antibody function (○——○) during the primary immune response against horseradish peroxidase emulsified in FIA. Kinetics of proliferation of immunoglobulin-producing cells in control rats immunized with FIA emulsified with PBS (×——×). Each point represents the arithmetic mean of the number of positive cells per lymph node detected separately in at least three different rats.

rapidly and plateaued until day 25 and subsequently they gradually diminished.

Before any antibody-synthesizing cells were detectable, cells synthesizing immunoglobulin without detectable antibody function appeared 3 days after antigen injection. These cells increased more rapidly than the antibody-synthesizing cell population. Ig-synthesizing cells were more numerous than antibody-producing cells until day 17 while after this more antibody- than immunoglobulin-synthesizing cells were observed. The total number of cells containing antibody or Ig without antibody function was approximately the same (Fig. 1).

In animals injected with adjuvant alone, the popliteal lymph nodes enlarged and contained approximately 50 per cent of the number of cells found in lymph nodes stimulated with antigen plus adjuvant. Antibody-producing cells were never observed, but a small number of immunoglobulin-synthesizing cells was detected on days 5–15 after adjuvant injection (Fig. 1). The ratio between the number of cells synthesizing immunoglobulins without antibody activity in animals immunized with peroxidase in Freund's incomplete adjuvant and the number of immunoglobulin-synthesizing cells in animals immunized with adjuvant alone varied during the immune response; from 3 to 10 between days 3 and 8, rising to 10–30 between days 10 and 30, then falling somewhat to 5–10 several months later. Similar findings were obtained with Freund's complete adjuvant.

Fig. 2 represents the changes, after giving peroxidase, of the ratio between cells synthesizing immunoglobulins without antibody activity (immunoglobulin-synthesizing cells induced by the adjuvant alone being subtracted) and antibody-containing cells during the immune response. Mean values varied from 6 to about 200 between days 4 and 10, then

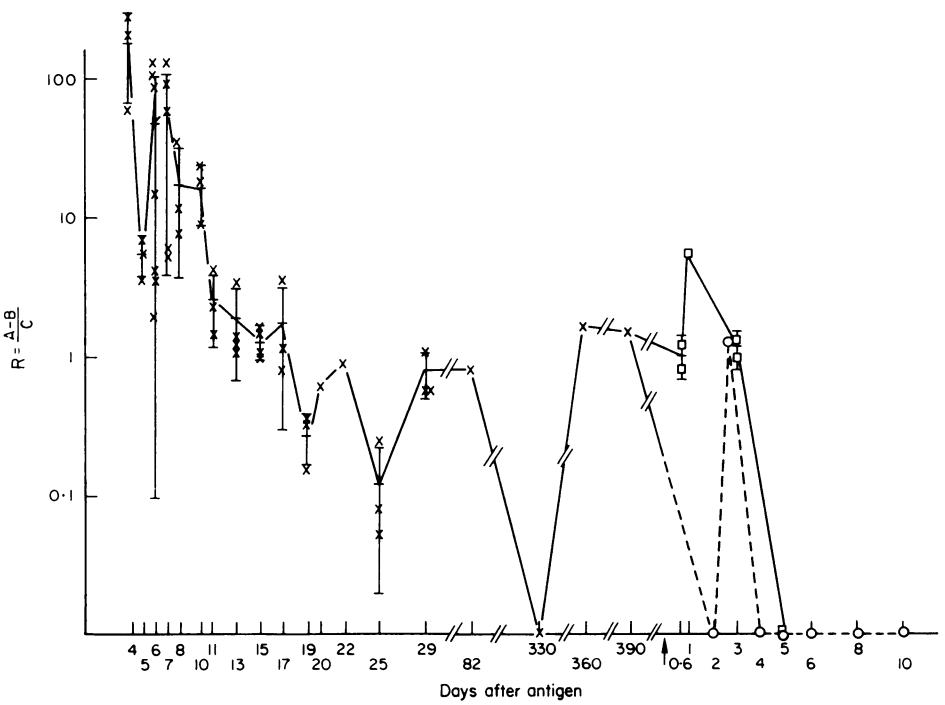

Figure 2. Ratio between cells producing immunoglobulins without detectable antibody activity but induced by the antigen- and antibody-synthesizing cells various times after primary and secondary stimulus. Each point represents the ratio obtained between a test rat and a control rat. Standard errors are shown by vertical bars. Arrow indicates the day of secondary challenge. A = cells synthesizing immunoglobulin without antibody activity in animals immunized with peroxidase; B = immunoglobulin-synthesizing cells in animals immunized with FIA; C = antibody-synthesizing cells. First injection: 1 mg of peroxidase emulsified in FIA (×——×). Second injection: 500 μg/ml of peroxidase given as a solution of PBS (□——□). Control rats received PBS only. Another group received 500 μg or 1 mg Po emulsified in FIA (○---○). Control rats received a second injection of FIA, emulsified with PBS.

gradually decreased to reach values of 1 at days 15 to 17. After this time, the ratio was below 1 and in some rats was 0. However, in about half of the rats observed late after primary immunization (3–13 months) it was observed that the ratio was slightly higher than 1.

Evolution of IgG- and IgM-producing cells

By using anti-Fcγ and anti-μ antibodies labelled with AP we found that IgG- and IgM-producing cells appeared simultaneously. Fig. 3 shows that between days 3 and 15, 25–40 per cent of the cells containing Ig were labelled with anti-μ chain antibody while 50–80 per cent of the cells were labelled with anti-Fcγ antibody. After this time the great majority of cells synthesized IgG, IgM-producing cells representing only 5–10 per cent of total Ig-positive cells. The distribution of the population of cells synthesizing Ig without antibody activity into IgG and IgM producers was not studied in detail, but at the beginning of the immune response, where there was a small number of antibody-containing cells (days 4–10), it is assumed that they were divided approximately as shown in Fig. 3.

Type of antibody- and immunoglobulin-producing cells

The different types of antibody-producing cells arising during the primary response were characterized.

At the beginning of the immune response, positive cells were plasma cells at different stages of differentiation. Staining was localized throughout the cytoplasm (Fig. 4a) or in some cells it was situated in a small area of the cytoplasm. This latter type was observed throughout between 4 and 30 days after injection. The intensity of the staining gradually increased. Between 20 and 30 days, the majority of the cells were intensely stained mature plasma cells, while at later stages (3–13 months) the positive cells observed could be classified in two types, highly differentiated plasma cells and small lymphocytes. The ratio between these two types was highly variable for each individual. In some rats lymphocytes represented 90 per cent of positive cells, in the other practically all cells were large plasmacytes. The morphology of cells synthesizing Ig without antibody activity has not been studied in detail. Only some characteristics observed at the level of the light microscope can be given: the majority of the positive

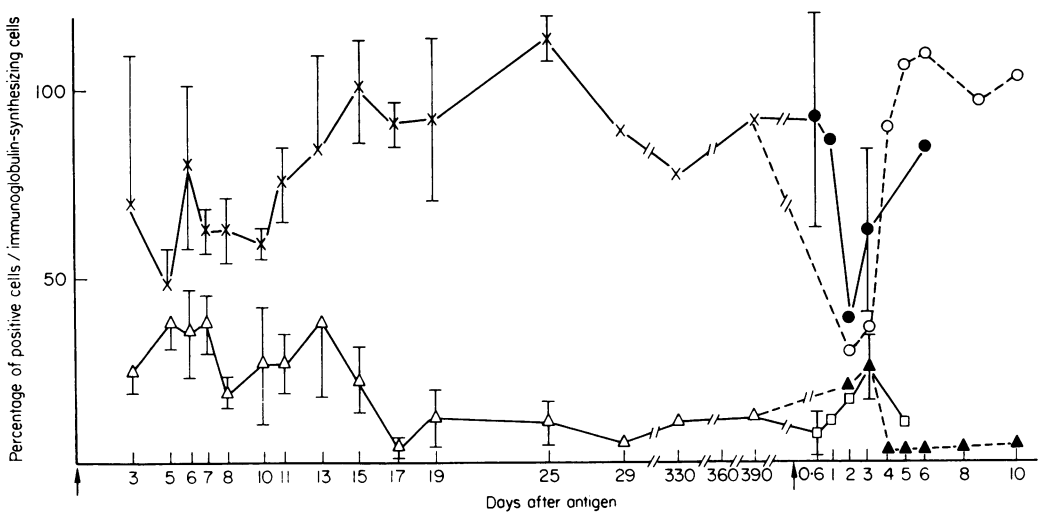

Figure 3. IgG- and IgM-synthesizing cells were respectively detected with monospecific anti-Fcγ and anti-μ antibodies labelled with alkaline phosphatase. The total number of Ig-positive cells taken as 100 per cent was determined with an anti-Fab antibody labelled with Po or AP. The percentage of each class was determined various times after primary or secondary stimulations with Po. Primary response: (×——×) IgG-synthesizing cells; (△——△) IgM-synthesizing cells. Secondary response: (●——●) IgG-synthesizing cells in animals boosted with soluble Po; (□——□) IgM-synthesizing cells in animals boosted with soluble Po; (○- - -○) IgG-synthesizing cells in animals challenged with Po in FIA; (▲——▲): IgM-synthesizing cells in animals challenged with Po in FIA. Arrows indicate primary and secondary challenge. Each point represents the arithmetic mean of results obtained in at least three different rats. Vertical bars represent standard deviations.

cells were plasma cells at different stages of maturity, and staining was always distributed throughout the cytoplasm (Fig. 4b): in some cells the Golgi apparatus was unstained.

Secondary response

Kinetics of proliferation of antibody and immunoglobulin-producing cells

Two parameters were tested during the secondary immune response: (1) the physical form of the antigen, i.e. solubilized in PBS or emulsified in Freund's adjuvant (FIA); (2) the time between primary and secondary injection.

Antibody-producing cells appeared 2–3 days following challenge. When Po was injected in FIA 12 months after primary injection (Fig. 5a) there was a very sharp increase between days 3 and 4 followed by a rapid disappearance of antibody-producing cells. If Po was inoculated as a solution in PBS about 1 year after primary stimulus, we observed at 5 days only one-tenth of the total number of antibody-containing cells obtained after challenge with Po in FIA (Fig. 5b). Approximately the same number of positive cells was obtained when rats were boosted with soluble Po or with Po in FIA 3 months after primary injection. Likewise, if Po was inoculated as a solution in PBS about 30 days after primary injection a very large number of antibody-synthesizing cells was observed early after the secondary stimulus (Fig. 5c). Therefore it can be concluded that some quantitative differences between the two physical forms of antigen are found only when a long time elapsed between the first and the second challenge.

Whatever the physical form of antigen and whatever the time elapsed between primary and secondary stimulus the absolute number of cells synthesizing immunoglobulins without antibody function was very low during all the secondary responses (compare Fig. 5a, b with Fig. 1). A slight increase of these cells was detected in the group of rats challenged with soluble peroxidase (Fig. 5b), and this population was practically absent after injection of Po in FIA (Fig. 5a). The ratio between the two populations, antibody- and immunoglobulin-producing cells, was measured, and on Fig. 2 we can see that early after the secondary stimulus the latter population was always present between 15 h to 3 days then totally disappeared at 4–5 days and later.

Evolution of IgG- and IgM-producing cells

The proportion of Ig-containing cells producing IgG and IgM was also studied in the secondary response. In Fig. 3 one can see that there was a rapid appearance of IgM-producing cells. These cells disappeared rapidly and by 4–5 days were totally replaced by IgG-producing cells. From Fig. 3, it can be determined that at days 2 and 3, cells synthesizing other classes than IgG2 or IgM were present when the booster was Po in PBS or in Freund's adjuvant.

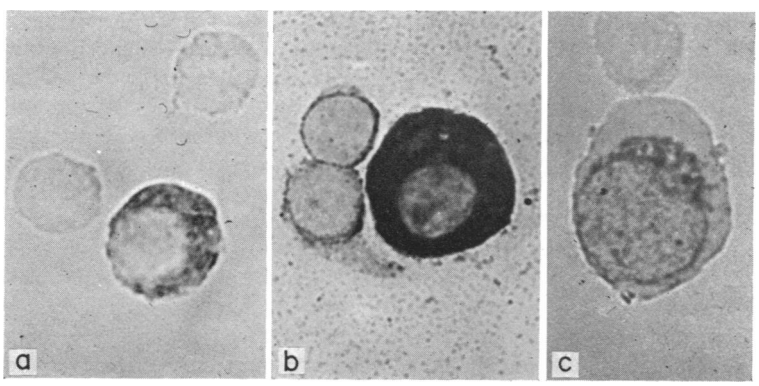

Figure 4. (a) Antibody-synthesizing plasma cell from popliteal lymph node removed 8 days after primary stimulation with Po emulsified in FIA. Cells were incubated for 1 h with a 100 μg/ml solution of peroxidase, then after washings, stained with Graham and Karnovsky's substrate. Staining is localized throughout the cytoplasm. (b) Immunoglobulin-synthesizing plasma cell from popliteal lymph node removed 8 days after primary stimulation with Po emulsified in FIA. Cells were incubated for 3 h with a 250 μg/ml solution of anti-Fab antibody labelled with AP and stained. Staining is localized throughout the cytoplasm. (c) Antibody-synthesizing blast cell from popliteal lymph node removed 2 days after secondary challenge of Po in FIA and 12 months after primary stimulus. The staining is localized in the perinuclear space and in some compartments of the cytoplasm (Golgi apparatus or endoplasmic reticulum).

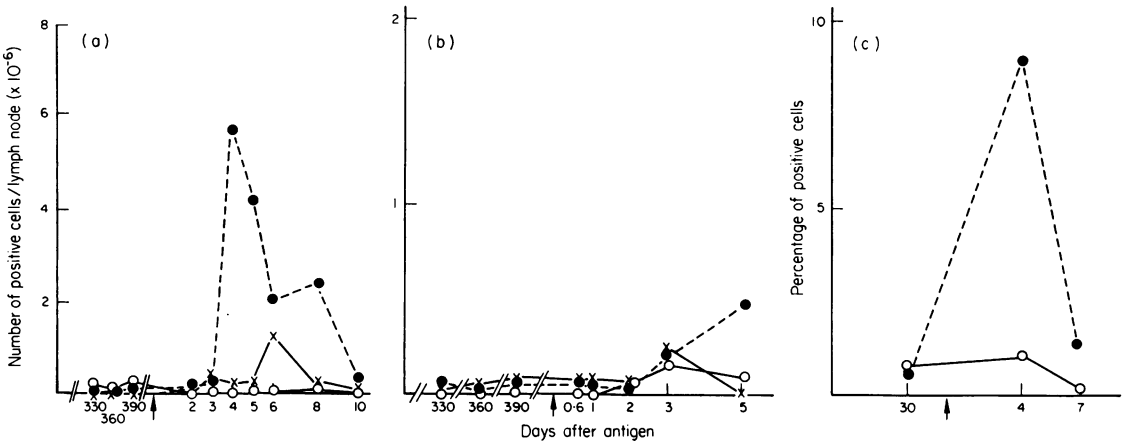

Figure 5. Kinetics of proliferation of antibody-synthesizing cells (● – – – ●) and of cells synthesizing immunoglobulins without antibody activity (○——○) during the secondary immune response. (a) About 1 year after primary stimulus, rats received a booster injection of 500 or 1000 μg of peroxidase emulsified in FIA. Kinetics of proliferation of immunoglobulin-synthesizing cells were also studied in animals boosted after 13 months with FIA emulsified with PBS (×——×). (b) Group of rats which received a booster injection of 500 μg of soluble peroxidase about 1 year after primary inoculation. Kinetics of proliferation of immunoglobulin-synthesizing cells was also studied in animals boosted after 13 months with PBS alone (×——×). (c) This group of animals received early (32 days) after primary stimulus a second injection of 0·5 mg soluble peroxidase. Arrows indicate the time of secondary challenge.

Type of antibody-producing cells

Lymph nodes removed early after booster injection (15 h–24 h) contained many cells with endogenous peroxidase activity (macrophages, eosinophil polymorphonuclears). Most of the early antibody-producing cells were less differentiated than those observed initially after primary injection. In some of them, especially in undifferentiated cells with a large cytoplasm and nucleus (Fig. 4c) antibody was visible in the perinuclear space. After 4–5 days positive cells became well differentiated plasmacytes with the heavy staining distributed throughout the cytoplasm.

DISCUSSION

The purpose of the present work was to study the two populations of immunocytes arising after antigenic stimulation: one synthesizing immunoglobulins without detectable antibody function and the other synthesizing specific antibodies.

Ig without detectable antibody function could be antibodies directed against impurities present in the preparations of antigen used. This was unlikely since the antigen injected was always very pure (tyrosinase, peroxidase, bovine serum albumin), as determined by immunoelectrophoresis and double immunodiffusion. Moreover, the same preparation was used for both primary and secondary injections. This phenomenon is not restricted to a single antigen but has also been described for other proteins: BSA (Urbain-Vansanten, 1970), tyrosinase (Antoine *et al.*, 1973), tobacco mosaic virus (Urbain-Vansanten, 1970), sheep red blood cells (Moticka, 1974), and in three different species: rabbits; mice; rats. Similar results were also obtained whatever the adjuvant used: Freund's complete or incomplete adjuvant, indicating that the Ig without antibody function did not represent antibodies directed against mycobacteria. Moreover, antibody-producing cells and cells synthesizing Ig without antibody activity were also observed after stimulation of animals with different antigens injected as a solution in PBS (Antoine, Petit and Avrameas, unpublished results).

Attempts to increase the sensitivity of the methods used to detect the antibody-containing cells did not change the results. Whatever the direct or indirect procedures used on fixed or unfixed preparations, it was not possible to obtain the same percentage for antibody-containing cells and for cells synthesizing Ig without antibody activity. Thus is it unlikely that these latter cells contained a small amount of active antibodies.

It has been proposed that antibodies could be

directed against antigenic determinants only accessible after partial degradation or denaturation of the antigen molecule (Scibienski, 1973) and thus impossible to detect when native antigen is used for staining as in our experiments. This leaves unexplained the differences between primary and secondary responses reported here. It is known that diverse substances can activate non-specifically a large part of the immune system (Rubin and Coons, 1971; Coutinho and Möller, 1973; Coutinho, Möller, Andersson and Bullock, 1973). It was suggested that stimulated T cells liberated some non-specific substances possessing activating properties capable of inducing a polyclonal antibody response. If such substances are released by antigen-stimulated T cells, it is possible that they induce polyclonal proliferation and differentiation of many cells bearing specificities unrelated to the antigen injected. In the secondary response, however, where only antibody-producing cells were detected, the same explanation could not apply.

It is also unlikely that all the tested antigens possessed some unspecific mitogenic properties for B cells. Preliminary experiments have shown that when peroxidase was injected into thymectomized, irradiated and bone-marrow restored mice, both populations of cells were absent suggesting that peroxidase did not act as a B-cell mitogen (Antoine and Avrameas, unpublished results).

A hypothesis we are now trying to test is that the Ig without detectable antibody function are in fact very low affinity antibodies. This hypothesis, compatible with the theory expressed by Siskind and Benacerraf (1969) is difficult to sustain from the recent works published by Werblin, Kim, Quagliata and Siskind (1973) and by Kim and Siskind (1974) who have shown that low affinity antibodies were present in approximately the same amounts throughout the period of the primary immune response studied. These results fail to explain the sequential appearance of our two populations. Moreover, some recent studies published by the above-mentioned authors indicated that the 'maturation' of the immune response greatly varied with the adjuvant used (Mond, Kim and Siskind, 1974). In our experiments, the sequential appearance and the size of the two populations of cells we studied did not change whatever the adjuvant utilized. Finally Sidorova, Chernomordik and Gurvitch (1975) have shown that there is no interaction between antigen and Ig without antibody activity.

From the above discussion it is evident that our results are difficult to explain with any of the previously described hypotheses. An alternative one is therefore proposed.

Previous papers from our laboratory reported that there are some striking cytological differences between primary and secondary responses suggesting that these two events of the immune response differ not only in the quantity of antigen-reactive cells but also in other fundamental ways (Kuhlmann and Avrameas, 1972, 1975; Miller, Avrameas and Ternynck, 1973a, b).

It was concluded that in the primary response, differentiation of immunocytes responding to the antigenic stimulation preceded antibody synthesis, but in the secondary response differentiation and antibody synthesis are simultaneous events. The most intriguing observation was that some cells contained antibody in few cisternae or in the Golgi apparatus, and were also labelled in the remainder of the cytoplasm with enzyme-conjugated anti-Ig antibody indicating that the other cisternae were filled with material possessing the antigenic determinants of Ig (Miller *et al.*, 1974, 1975).

The present paper shows that a population of cells synthesizing Ig without antibody function appeared and reached a maximum before the antibody-producing cells. The kinetics of the two populations, apart from the timing, and the total size of the two populations were rather similar. In the secondary response where differentiation and antibody synthesis were simultaneous, only antibody-forming cells were detected in significant numbers. Thus it seems plausible that Ig-synthesizing cells are precursors of antibody producers, suggesting that in the primary response animals are unable to directly produce antibody with measurable affinity for antigen and this step is preceded by a period characterized by proliferation, maturation and synthesis of Ig. Some biosynthetic or genetic mechanisms must also take place in cells, to allow in the secondary response a rapid production of antibodies with detectable function. We are now trying to determine the nature of the Ig without antibody function, particularly if this Ig is closely related to the antibody or not.

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