ELECTROPHORETICALLY HOMOGENEOUS ANTIBODY SYNTHESIZED BY SPLEEN FOCI OF IRRADIATED REPOPULATED MICE*

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Studies in the rabbit have shown a focal constitution of the stimulated lymph node. In fact, small lymph node fragments, when incubated in vitro individually, synthesize electrophoretically homogeneous immunoglobulins. Electrophoretic bands were shown to be homogeneous also with regard to class, allotype, and antibody specificity (1). These results were essentially confirmed for spleen fragments of mice immunized with sheep red blood cells (Luzzati and Herzenberg, unpublished results). A possible interpretation of these findings is that the normal antibody response derives from a large but finite number of functional units composed of homogeneously differentiated cells. In this case, the study of the product of single units would give some insight into the events which bring about the initiation of the immune response. However, in the normal lymphoid tissue the units apparently overlap and cannot be separated. A way of overcoming this difficulty seems to be the study of the product of the foci of antibody synthesis obtained by repopulating irradiated mice. In fact, as previously described by several authors, injection of a limiting number of lymphoid cells in irradiated mice elicits, upon antigenic stimulation, the appearance in the spleen of discrete foci of specific antibody activity. These results have been obtained using both unprimed and primed cells (2-7). A linear relationship is consistently obtained between number of foci per spleen and number of cells injected, thus indicating that the foci represent independent immunocompetent units. Therefore, these foci seem to provide the most suitable system for the study of the events involved in the initiation of immunological response. Up to now, many attempts have been made in order to elucidate the number and type of cells participating in the formation of the immunocompetent units and the mechanism of their interaction (8–12). Moreover, the characterization of the product of the foci has been pursued, mostly from the point of view of the class of Ig produced (5, 13, 14). These experiments were always performed with modifications of the Jerne technique, looking at either direct or indirect plaques. However, these approaches do not allow the isolation and the structural study of the products.

Our approach, reported in this paper, was to excise single foci from the spleen and to make them synthesize antibody in culture in the presence of labeled amino acids. The product was subsequently isolated and analyzed by immunochemical methods (Fig. 1).

Materials and Methods

Animals.—Male mice of the strains CWB, CSW, and C3H were employed.

Immunization and Cell Transfer.—Donors were immunized by three weekly injections of 5×10^8 sheep red blood cells (SRBC) intraperitoneally. 1 wk after the last injection they were sacrificed, the spleens aseptically removed and teased to make a cellular suspension. Cells from three spleens were pooled, washed once with Hanks' solution, and counted. Recipients were exposed to 550 R X-irradiation (irradiation conditions: Philips X-ray machine set at 250 kv, 15 ma. Inherent filter 1. 0 Al; added filter 0.25 Cu. At a distance of 65.5 cm and 80 R/min radiation rate). In some experiments a dose of 900 R was given. Within 4 hr after irradiation, the recipients were given 50 U heparin intraperitoneally and then were injected in the tail vein with 10^6 nucleated spleen cells and 5×10^8 SRBC in 0.2 ml total volume.
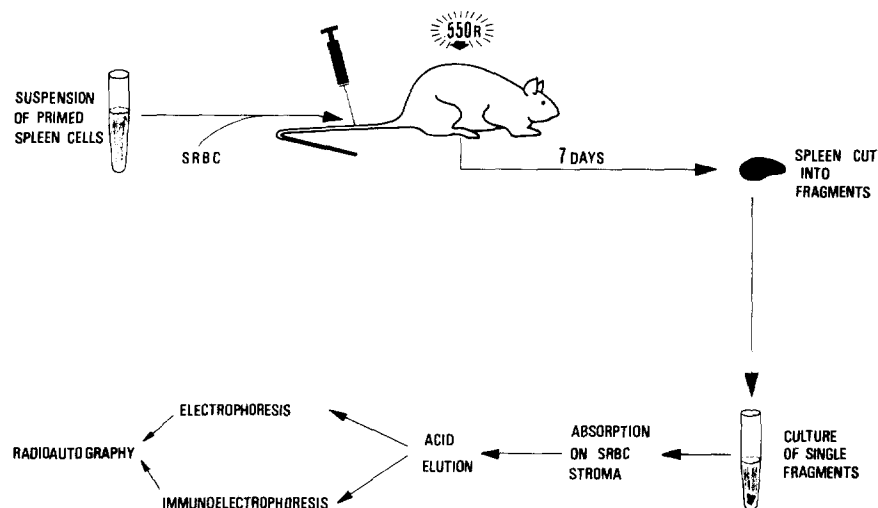

Fig. 1. Schematic outline of the experimental procedure.

In Vitro Cultures.—8 days after the cell transfer, the recipients were sacrificed and the spleens were aseptically removed and placed in cold NCTC 109 medium (Microbiological Associates, Inc., Bethesda, Md.) without leucine, lysine, arginine, and valine. The spleen was then sliced and each slice was consecutively numbered. About 12–16 slices were obtained from a spleen. Each slice was cut into 2–6 fragments which were also labeled to keep track of their location. In this way, the position of each fragment in the spleen could be mapped.

The culture procedure essentially followed the one described previously (1). Each fragment (1–1.5 mg wet weight) was incubated in vitro in 0.3 ml of the same medium as above, supplemented with 20% fetal calf serum and 2 μCi/ml each of ^{14}C leucine (specific activity, 222 mCi/mm), lysine (specific activity, 214 mCi/mm), valine (specific activity, 185 mCi/mm) and arginine (specific activity, 222 mCi/mm). Incubation was carried for 24 hr with constant shaking in an atmosphere of 95% O_2 and 5% CO_2, after which the supernatants were collected. The antibody activity present in each culture fluid was tested by agglutination of SRBC.

Preparation of SRBC Stroma.—The method followed was essentially that described by Brown and Ellis (15). The final stroma suspension was standardized on the basis of protein content. This was determined with the Lowry method (16). A longer (1 hr) treatment with the

alkaline copper reagent was necessary in order to completely dissolve the stroma. A linear relationship was obtained between optical density at 700 mμ and amount of stroma suspension. The preparation was stored at $-20°C$. After thawing, 1:10,000 merthiolate was added and then the suspension was kept at 4°C.

Absorption and Elution of Anti-SRBC Antibodies.—Absorption of the antibody synthesized in culture was performed by adding to 200 μliters of the culture fluid a 10-fold excess of the minimum amount of stroma necessary to remove all agglutinating activity. Incubation was carried on for 1 hr at 37°C followed by 12 hr at 4°C. The stroma were sedimented and washed twice with 1 ml of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) by centrifugation at 1900 g at 4°C for 3 hr.

The pellets were then carefully resuspended in 40 μliters of 0.47 M glycine-sulfate buffer pH 2.5. After 5 min at room temperature, the suspension was centrifuged at 10,000 g for 10 min in the cold. 30 μliters of supernatant were transferred to small plastic tubes (LP/2, Luckham Ltd, Sussex, England) containing 7 μliters of 1 M Tris(hydroxymethyl)aminomethane-HCl, pH 10. The resulting pH was 7.3–7.4.

5 μliters of normal mouse serum were added and the eluate was dialyzed against Veronal buffer, pH 8.6, 0.1 M for 30 min, by covering the mouth of the tubes with dialysis membrane. This procedure was adequate to avoid distortion of the electrophoretic run because of the too high salt concentration. The volumes of the eluates were not appreciably changed after dialysis.

Electrophoresis.—High voltage agar electrophoresis was performed according to Wieme (17), using 25 μliters of material. Six samples were run on the same 8 $\times$ 10 cm slide. After fixing and washing, the slide was dried and stained.

Immunoelectrophoresis.—The micromethod of Scheiddegger (18) was employed using 5 μliters of dialyzed eluate after further addition of 2 μliters of immune mouse serum as carrier. A rabbit anti-mouse serum, giving strong precipitin lines with IgG and IgM, was added to the troughs. After 24 hr, the slides were extensively washed, then dried and stained.

Radioautography.—Electrophoresis and immunoelectrophoresis slides were overlaid with a Kodak Super-XX Panchromatic cut film. After 15–20 days exposure, the films were developed with Kodak D 76 developer and fixed with the Kodak Unifix.

RESULTS

Protein Synthesis by Spleen of Irradiated Mice.—Irradiated, nonrepopulated mice were injected with 5 $\times$ 10⁸ SRBC. Under these conditions, spleen fragments did not show any in vitro synthesis of specific antibody. However, when the culture fluids were subjected to high voltage agar electrophoresis followed by radioautography, the patterns obtained showed a variable number of bands in beta-gamma region. Also the anodic of the electropherogram showed some radioactive products, although in a less defined pattern. The banding in beta-gamma region was clearly due to Ig. In fact it disappeared upon treatment with a rabbit anti-mouse Ig serum, which left essentially unaffected the radioactivity in the other regions (Fig. 2).

The immunoelectrophoresis developed with the same antiserum showed that IgG and IgM were present in variable proportions. On the hypothesis that this amino acid incorporation by the host spleen is due to an insufficient radiation dose, animals were irradiated with 900 R in some experiments. However, even under these conditions, the same radioautographic patterns were obtained.

Specific Antibodies Produced by Spleen of Irradiated Repopulated Mice.—When irradiated mice were repopulated with 10^6 donor spleen cells and injected with 5×10^8 SRBC, only some of the fragments synthesized specific antibody. The hemagglutination titers ranged from undiluted to 1:128. Checking back the location of the active fragments in the spleen, they were found to be clustered in well distinct groups surrounded by negative tissue. The number of active areas per spleen varied between one and four with an average of 2.4. Sometimes the boundaries of single foci were not clearly defined and in these cases the assessment of the precise number of foci was difficult. However, in most cases such as the ones represented in Figs. 4, 5, and 6, the areas were so well localized that there could be little doubt that they represented single foci.
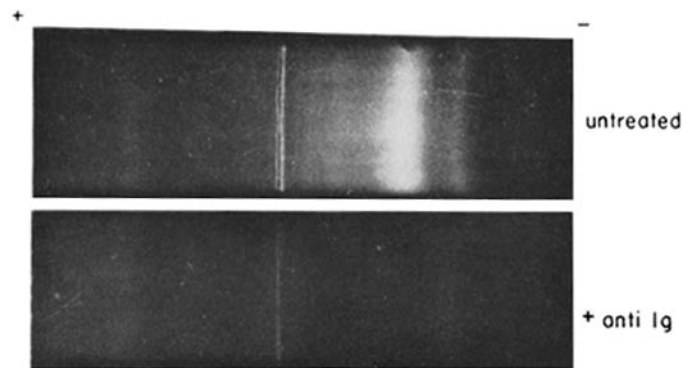

FIG. 2. Radioautographic pattern of a culture fluid untreated and after treatment with a rabbit anti-mouse Ig serum.

The culture fluids were directly subjected to high voltage electrophoresis followed by radioautography. A pattern of more or less defined bands was obtained. However, there was no appreciable difference between the patterns from fragments coming from either positive or negative areas. The absorption of culture fluids with enough SRBC to completely remove the agglutinating activity either did not affect the radioautographic pattern or, more rarely, caused a weakening of some bands in beta-gamma region. However, also in these cases, the high background left after absorption did not allow a clear interpretation of the results. Presumably, the Ig synthesis of host origin described above is of such a degree as to obscure the synthesis of specific antibody by cells of donor origin. Therefore, the isolation of specific antibodies from single foci was attempted through absorption on SRBC stroma and subsequent elution.

In preliminary experiments, it was found that 8 μg of stroma proteins were able to remove all agglutinating activity from 200 μliters of a pool with a titer 1:64 obtained by mixing 10 culture fluids. It was decided to use a 10-fold excess

of this amount for every culture fluid, independent of its titer. The effectiveness of the absorption was checked by titration of the supernatants. Under these conditions, about 10% of the nondialyzable counts were absorbed on the stroma. Two washings with 1% BSA in PBS were adequate to remove all nonbound or nonspecifically bound radioactivity. The acid elution with glycine-sulfate buffer removed about 50% of the ^{14}C counts. Further elution removed only a few additional counts. Therefore only the first eluate was used throughout this work.

The specific antibody activity of the eluate was confirmed by its ability to agglutinate SRBC (about half of the original agglutinating activity was recovered) and to bind again to antigen (more than 80% of the counts are reabsorbed on the stroma). Therefore, the product finally run on high voltage agar electrophoresis and immunoelectrophoresis represented a concentration of about four-fold in term of volume and a two-fold enrichment in terms of specific antibody when compared with the original culture fluid.

The radioautographic patterns after agar electrophoresis of the eluates showed a considerable degree of homogeneity. Distinct and sharp spikes were localized only in the beta and gamma region. In some eluates from fragments coming from the same single focus, a band in alpha-2 region near the origin was present. Some examples of the electrophoretic patterns observed are given in Fig. 3. It is clear that antibodies purified from different culture fluids give a unique pattern of mobility, intensity, and number of radioactive bands.

On the other hand, the eluates derived from stroma coated with a pool of 20 different active culture fluids gives, in electrophoresis, a diffuse smear of radioactivity in beta-gamma region. In no case, did culture fluids devoid of agglutinating activity give any radioactive pattern in electrophoresis when subjected to the same procedure.

In the Figs. 4, 5, 6, some spleens are shown in which the foci were clearly definable as single units, being composed by few active fragments and being surrounded by large areas of negative tissue. The single focus present in the spleen of Fig. 4 synthesized an antibody which migrated as a single sharp band. The same statement can be made for the foci 130 and 141 + 143 of the spleen shown in Fig. 6. In the case of focus 141 + 143, the same band is produced by two adjacent fragments belonging to the same focus. On the contrary, the single focus present in the spleen of Fig. 5 undoubtedly produced two distinct bands, both in gamma-1 region and with about the same intensity. A second component is also present in the focus 150 + 152 of Fig. 6, although of lower intensity compared to the major spike. In some other cases, not shown in the figures, three and sometimes four bands are present, but in these instances the possibility of confluence between different foci cannot be ruled out.

In immunoelectrophoresis, the radioactivity was localized exclusively either in IgM or IgG lines or both, varying from one focus to another. Generally, the lines were not uniformly labeled in all their length; a good correspondence exists
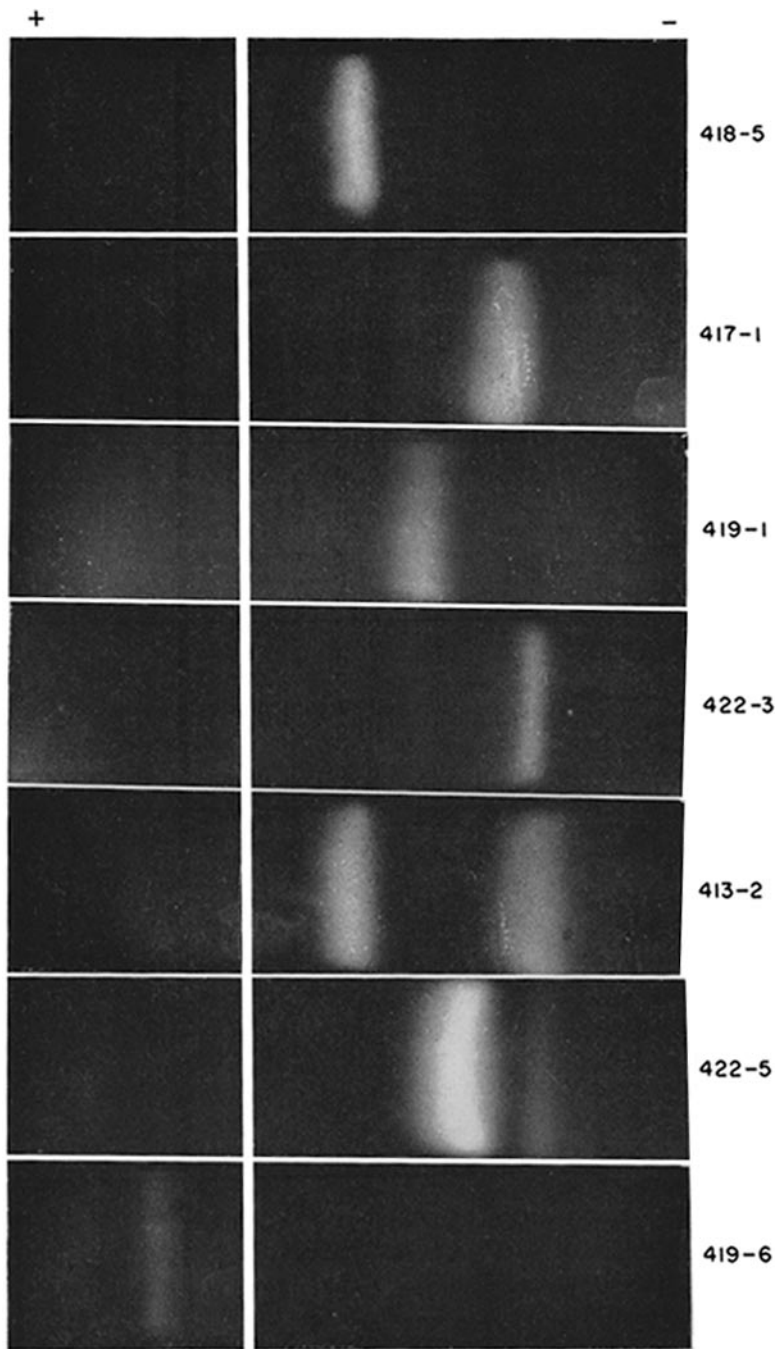
Fig. 3. Radioautographic pattern of antibodies produced by different single fragments and eluted from SRBC stroma.

between the position of spikes in electrophoresis and of radioactive stretches in immunoelectrophoresis. In some cases, when two bands are present, they appear
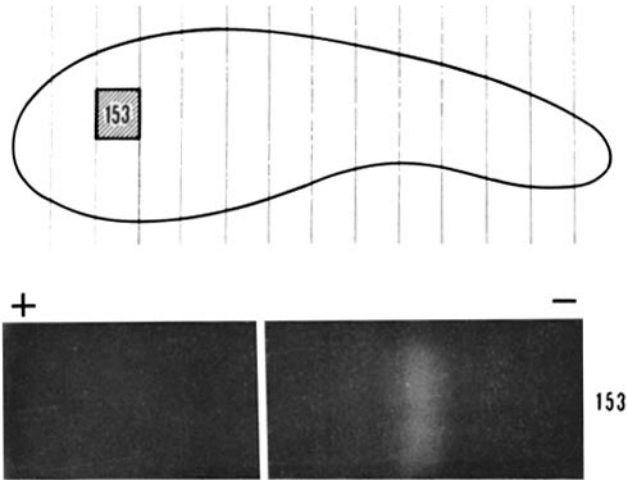

FIG. 4. Spleen 24-4: localization of active areas within the organ and radioautographic pattern of the antibody eluted from SRBC stroma.
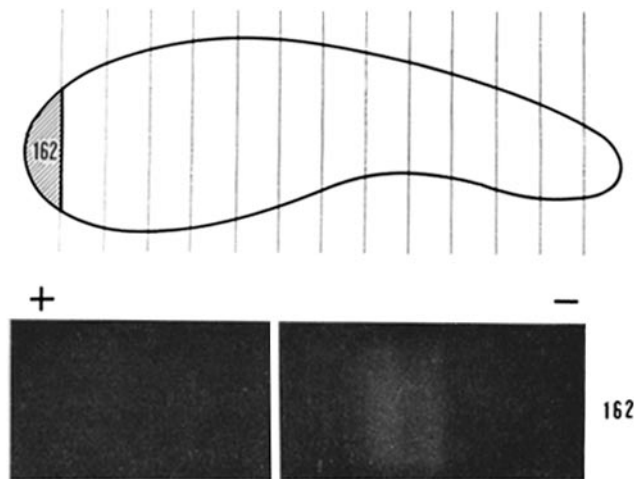

FIG. 5. Spleen 30-4: localization of active areas within the organ and radioautographic pattern of the antibody eluted from SRBC stroma.

to belong, one to IgG, and the other to IgM class, as in the instance shown in Fig. 5. In other cases, two bands are present, both belonging to IgG class. In these instances, when the electrophoretic bands differed considerably for their
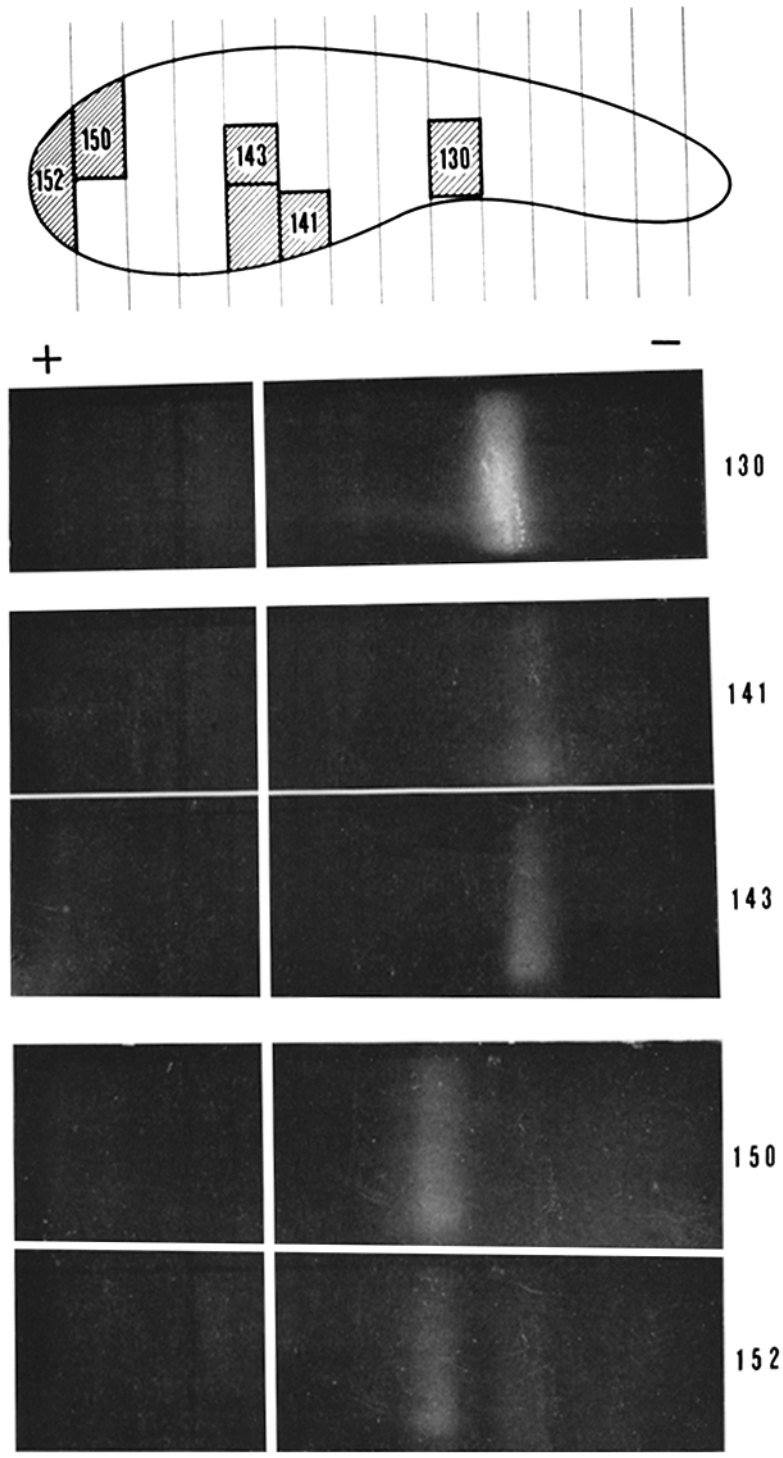
FIG. 6. Spleen 30–3: localization of active areas within the organ and radioautographic
pattern of the antibody eluted from SRBC stroma.

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mobility, in immunoelectrophoresis a characteristic pattern is apparent, two radioactive stretches adjoined by a barely visible connecting bridge.

DISCUSSION

The results presented in this paper show that this experimental approach allows the localization of foci of antibody activity in irradiated mice repopulated with a limiting number of lymphoid cells. Under this respect, this procedure can be compared with the methods described by other authors (2, 3, 6) with the additional advantage that the product is labeled and can be isolated and analyzed.

A serious difficulty in the analysis of the product of foci was the residual immunoglobulin synthesizing activity present in the host spleen after irradiation. This could be explained by the known radioresistance of plasma cells in comparison to other cells present in the lymphatic tissue (19, 20). It is noteworthy that this background of Ig activity shows a pattern of bands, which suggests that the focal constitution of the normal lymphoid organ (1) is not completely disrupted by the radiation damage.

· The acid elution procedure proved to be adequate to remove all background radioactivity. Since most of the eluted material was reabsorbable on the stroma and possessed agglutinating activity, it can be affirmed that there was little or no denaturation caused by the acid treatment. It can be argued that the elution procedure causes a selection in the population of antibody molecules on the basis of different affinity, for instance. Although this possibility cannot be excluded, the relatively high recovery makes it unlikely that it could produce a gross distortion of our results.

Our data show that the antibody produced by single foci has a very high degree of homogeneity, as judged by electrophoretic mobility when compared with the antibody present in the serum of immunized nonirradiated animals. In agreement with these findings, it has been recently reported (21) that the antibody produced by single foci presents a high degree of functional homogeneity in its capacity to bind antigen.

In some instances, the homogeneity is complete, i.e., a single protein is synthesized by a single focus, as shown, for example, in Fig. 4. This situation recalls the homogeneity of myeloma proteins, which, although they may show antibody activity, are produced by neoplastic cells which multiply free from control. On the contrary, the spleen foci are antigen-dependent immunological units, even though the conditions in which they are obtained cannot be regarded as completely physiological.

Our results show that all cells produced by the clonal expansion which characterizes the initiation of the immune response (22) may be uniformly differentiated toward the synthesis of one and the same molecular species. Thus, the same kind of differentiation which is commonly recognized as typical of single plasma cells may be a property of whole clones.

However, foci producing at least two proteins with different electrophoretic mobility were encountered with such a frequency as to practically exclude the possibility that they may arise from the overlapping of different foci, each producing a single molecular type of antibody. This finding may have two alternative explanations: either the differentiation pattern changes during the clonal expansion, or more than one precursor of antibody-forming cells (PC) participates in the formation of a single immunocompetent unit.

These alternatives can be considered in the light of the present views on the initiation of the immune response. In fact, the possibility has been put forward (8–12) that a focus represents the result of the interaction between one thymus-derived antigen-reactive cell (ARC) and one or more bone marrow-derived cells; after this interaction the latter proliferate and differentiate into antibody-forming cells. This hypothesis has been implied mainly for the primary response, however it seems likely that the mechanism underlying the secondary response is basically similar.

If our results are considered in the light of the above hypothesis, the following interpretation can be advanced: a focus producing a single molecular species could be derived from a single PC interacting with one ARC. The possibility of a one to one interaction has been directly verified by Saunders (23) who, by a modification of the Marbrook's in vitro system, found one PFC per focus until 18 hr of incubation.

Foci producing two or more bands could be derived from the interaction of one ARC with two or more PC. Thus, a focus would include more than one clone, each clone producing an individual antibody species. An alternative possibility is that a single PC is, even in these cases, involved in the formation of the focus, but during the clone expansion, a switch in the differentiation pattern may occur. The fact that two bands belonging to the same focus are in some cases IgM and IgG can be interpreted as examples of foci caught during the process of switching from IgM to IgG class production. However, other foci show two bands, both belonging to IgG class.

A firm choice between the two hypotheses cannot presently be made on the basis of our data. An answer could probably be reached by analyzing, by means of our procedure, foci produced by injecting a limiting number of thymus-derived cells, together with a large excess of bone marrow-derived precursors. Under these conditions, if multiple interaction sites exist in ARC (8, 24) they should be expected to be completely saturated and, therefore, multiple bands should consistently appear.

SUMMARY

10^6 splenocytes from primed donors were injected, together with sheep erythrocytes (SRBC), into X-irradiated syngeneic mice. 8 days later the spleens were excised and cut into small fragments, keeping track of their location in

the organ. Each fragment was cultured individually for 24 hr in the presence of ^{14}C amino acids and the culture fluids were assayed for antibody activity. The antibody-producing fragments were found to be clustered in few restricted areas (foci) surrounded by negative tissue.

The anti-SRBC antibody from single foci was purified by absorption on stroma followed by acid elution. Thereafter, it was subjected to electrophoresis and immunoelectrophoresis. The radioautography of the runs showed a considerable degree of homogeneity. Distinct and sharp spikes were localized in the beta and gamma region. The pattern of each focus is unique from the point of view of the number of spikes and their mobility. Eluates obtained from many pooled fragments gave a broad radioactive smear in beta-gamma region.

Many foci synthesized antibody migrating as a single band. This homogeneous protein is probably the product of a clone of cells homogeneously differentiated. However, some foci producing two and probably more antibody bands were also encountered. Two interpretations of the finding can be given. Either more than one precursor may participate in the formation of a focus or a differentiation switch may occur during the clonal expansion.

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