STUDIES ON THE COMPETENCE OF SINGLE CELLS TO PRODUCE ANTIBODIES OF TWO SPECIFICITIES

By HARRIET GERSHON, SARA BAUMINGER, Ph.D., MICHAEL SELA, Ph.D., and MICHAEL FELDMAN, Ph.D.

(From The Weizmann Institute of Science, Rehovoth, Israel)

(Received for publication 27 March 1968)

One of the prime problems of immunology today deals with the potential of the immune competent cell to produce antibody of a given specificity. In studying this problem, we are faced with three principal possibilities: (*a*) The competent cell has a limited amount of information in its genome which confers upon it the ability to synthesize antibody to one or a very few antigenic determinants (1–5). (*b*) The cell competent to produce antibody is omniscient; that is, its genome codes for all necessary sequences observed in the varied antibody response of the organism (6, 7). (*c*) The immune competent cell does not possess in its genome any information for specific antibody, and the specificity is conferred in an "instructional" manner, at a higher (transcription, translation, etc.) level (8).

One possible approach to differentiate between the first mechanism and the two others is the study of antibody formation by single cells. Evidently, if after immunization with two arbitrarily chosen antigens, each immune competent cell can form antibodies of more than one specificity, this will exclude the first possibility as the cellular basis for antibody production. A number of methods for studying antibody formation by single cells have been applied to test the predictions of the various theories of antibody production. These include assays for bacterial immobilization and agglutination in microdroplets (9, 10), phage neutralization in microdroplets (11),[1] fluorescent antibody (12–15), and the hemolysin plaque technique (16). In these various studies from 0 to 45% of the responding cells have been reported to produce antibody of more than one specificity.

In the present study we have used a modification of the Jerne hemolysin plaque technique (17) which facilitates the possible detection of cells producing antibody to two individual antigenic determinants and enables the screening of a high number of antibody-producing cells. In these studies we used erythrocyte antigens as well as an artificial antigen composed of a protein to which two chemically defined and non–cross-reacting haptenic groups were coupled. This antigen was human serum albumin to which polyalanyl and *p*-azobenzenearsonate groups were chemically attached.

[1] Mäkelä, O. Personal communication.

Materials and Methods

Studies with Erythrocyte Antigens.—

Animals: C3H female mice, 2.5–3 months of age, were used in the present study.

Erythrocytes: Whole blood samples, obtained from rabbits and chickens by cardiac puncture and from camels and sheep by bleeding of the carotid artery, were maintained in Alsever solution at 4°C (18). Before use, all erythrocyte (RBC) samples were washed three times with a minimum of eight volumes of saline (0.9% NaCl) per wash. RBC in saline were counted in a hemocytometer and diluted to the required concentrations.

Immunization and cell suspensions: Each experiment was composed of three groups of mice; two groups of singly immunized mice were injected i.v. with $4 \cdot 10^8$ RBC in 0.4 ml saline from one or the other of the two antigens in the pair tested; the doubly immunized group of mice received a single i.v. inoculum containing $4 \cdot 10^8$ RBC from each of the two species tested in 0.4 ml saline.

4 days after immunization, the mice were killed by cervical dislocation and their spleens were surgically removed. Cell suspensions were obtained by passing each spleen through a stainless steel mesh of pore size 0.6 × 0.6 mm into Hanks' balanced salt solution without bicarbonate. The spleen cell suspensions thus obtained were passed through 27 gauge needles to ensure complete dispersal of the cells, and appropriate dilutions were made for plating in the Jerne plaque assay. Each spleen cell sample was plated in three 10-fold serial dilutions to ensure plating of a dilution which would provide adequate numbers of sufficiently dispersed plaques. Spleen cells were counted in Turk's solution (0.5 ml of 1% gentian violet in distilled water, 1 ml of acetic acid, 100 ml of distilled water).

Hemolytic plaque assay: We used a modified version of the hemolytic plaque technique of Jerne et al. which facilitated microscopic scanning and provided plaques the diameter of which spanned the entire layer of agar used. This last was of critical importance in the present search for clean "double" plaques containing a single plaque-forming cell (PFC) in the center.

Molten Bacto-Agar (0.7%, Difco Laboratories, Detroit, Mich.) in Hanks' without bicarbonate (pH 7.1) plus 0.056% DEAE-dextran (Pharmacia, Uppsala, Sweden) was distributed in 1 ml aliquots into test tubes in a 45°C water bath. Just before plating, 0.1 ml of an appropriate dilution of spleen cells plus 0.1 ml of RBC suspension were added to each 1 ml of the agar. The tubes were removed from the bath and the contents rapidly mixed with a cyclomixer. The contents of each tube were spread in 0.3 ml aliquots onto microscope slides precleaned with 50% alcohol-ether. The agar, covering an area 5 × 2.5 cm, was allowed to set on the slides, which were then placed in moist chambers in a 37°C incubator for 1 hr. The slides were then flooded with 1 ml of a 1/25 dilution of guinea pig complement (Texas Biological Co., Ft. Worth, Tex.) in Kolmer saline (Kolmer Laboratories, Hendersonville, Tenn.) (8.5 g of NaCl, 0.1 g of $MgCl_2$, 1 liter of distilled water) and returned, in moist chambers, to the 37°C incubator for an additional 0.5 hr. Slides were then removed from the incubator and the number of plaques counted. Each sample of spleen cells was plated with three separate RBC backgrounds: with the two immunizing RBC, each on separate slides ($7.5 \cdot 10^6$ RBC per slide), and with an equal mixture of the two types of RBC together ($7.5 \cdot 10^6$ of each RBC per slide). Each kind of slide was plated in a minimum of two and a maximum of ten replicates to provide sufficiently large numbers of plaques for accurate counting.

Conditions and criteria for reading plaques: Slides were scanned and plaques counted under ×40 magnification; for closer scrutiny, ×100 magnification was used. In all cases a hemolytic area was called a plaque only if we could identify a central plaque-forming cell (PFC). In the case of "double" slides, i.e. those on which we seeded two types of RBC, our criteria for calling a plaque double were (*a*) the hemolysis of both types of RBC and (*b*) the presence of one and only one central PFC. We, therefore, performed each experiment with several dilutions of each

spleen suspension to ensure a sufficient scatter of cells. Throughout these readings the agar was prevented from drying by flooding with Hanks' solution.

Studies with Chemically Coupled Non-Cross-Reacting Haptens.—

Antigens: Two batches (1029 and 1041) of polyalanyl arsanil human serum albumin (pAlaArsHSA), which differed in the number and length of the polyalanyl side chains attached to the ϵ-amino groups of lysine residues, and *p*-azobenzenearsonate ("arsanil") groups linked to tyrosine and histidine residues of the HSA carrier, were used as antigens. The coupling was performed stepwise; first the "arsanil" groups were attached according to the method of Tabachnick and Sobotka (19), and subsequently the product was polyalanylated as described by Schechter et al. (20).

For the preparation of antigen 1041, 91 mg of arsanilic acid was dissolved in 1.26 ml of cold 1 N HCl. To this was added 29 mg of $NaNO_2$ in 2 ml of cold distilled water. The above mixture (solution I) was stirred well and maintained in the cold for 10 min and then quickly added to a solution containing 1 g of HSA in 12 ml of 0.25 M $NaHCO_3$ buffer, pH 9.5. The above reaction mixture was stirred overnight in the cold, dialyzed against distilled water, and lyophilized. Coupling of "arsanil" for the preparation of antigen 1029 followed the same procedure except that all the reagents in solution I were used in fourfold quantities.

To complete the preparation of antigen 1041, 1 g of the lyophilized ArsHSA (1041) was dissolved in 100 ml of cold 0.05 M phosphate buffer, pH 7, and mixed, at 4°C, with 1.5 g of *N*-carboxy-DL-alanine-anhydride (21) dissolved in 30 ml of absolute dioxane. This mixture was stirred overnight in the cold, dialyzed against distilled water, and lyophilized. For the preparation of antigen 1029 the same procedure was followed with the substitution of 1 g for 1.5 g of *N*-carboxy-DL-alanine-anhydride.

Determinations of the extent of enrichment of the protein derivatives with alanine residues (20) and the "arsanil" content (22) of antigens 1029 and 1041 were performed. Antigen 1029 contained on the average 6.45 *p*-azobenzenearsonate groups per molecule of HSA, 4.35 of these coupled to tyrosine residues and 2.1 to histidine residues. On the average, 83 alanine residues were bound to each molecule of HSA. The average length of the polyalanine side chain as determined by the deamination method used for polyalanyl ribonuclease (23) was 3 residues. Antigen 1041 carried 2.75 *p*-azobenzenearsonate groups per HSA molecule, 1.65 of these on tyrosine residues and 1.1 on histidine residues. The alanine content of antigen 1041 was 131 residues per carrier molecule, and the average number of alanine residues per chain was 4.5.

Animals, immunization, and source of cells: Randomly bred rabbits of both sexes, weighing 2–3 kg, were immunized intradermally in multiple lateral sites, the nape, and the rump, with 20 mg of antigen in complete Freund's adjuvant. On day 8 after immunization the rabbits were sacrificed with an i.v. injection of air. The inguinal, axial, and (or) brachial lymph nodes were removed and a cell suspension of pooled nodes was made as described above for mouse spleen. Appropriate dilutions of cells were plated in a modified Jerne technique.

Preparation of hapten-coupled erythrocytes: Sheep RBC, obtained by carotid artery puncture, were maintained in Alsever solution for a maximum of 72 hr before coupling of haptens, and used within 24 hr post coupling. The chemical attachment of "arsanil" groups to erythrocytes was performed with a modification of the method of Ingraham (24). With the quantities of reagents recommended by Ingraham we could not produce anti-arsanil plaques. We, therefore, used three times the arsanilic acid recommended by Ingraham for coupling to RBC and consistently produced large, clean plaques. SRBC were alanylated by a modification of the technique of Rimon and Sela (25) which entailed the use of 2 mg of *N*-carboxy-DL-alanine-anhydride for 0.1 ml of packed RBC and 0.1 M in place of 0.067 M phosphate buffer.

Hemolytic plaque assay: We followed the modified Jerne plaque assay outlined above with one additional alteration; the agar and DEAE-dextran of the original assay were replaced with

agarose (L'Industrie Biologique Française, Gennvilliers, Seine, France). Agarose improved the optics of the system and facilitated the search for clear "double" plaques by eliminating the turbidity in the agar caused by a precipitate formed with the DEAE-dextran. In preliminary experiments we also attempted the use of the Cunningham plaque technique (26). It was felt that this technique, with its single plane of RBC, would be ideal for detection of clear "double" plaques. We were disappointed, however, by the minute diameter of the plaques produced and, therefore, discarded the technique.

For each cell suspension assayed the following protocol was used: three 10-fold serial dilutions of immune cells in Hanks' solution were plated on Falcon bacteriological plastic Petri dishes (60 × 15 mm), following the original protocol of Jerne and Nordin (17) with the substitution of agarose for agar plus DEAE-dextran; each dilution of immune cells was plated in duplicate with SRBC, polyalanylated SRBC (pAlaSRBC), and arsanil SRBC (ArsSRBC) ($4 \cdot 10^7$ RBC per plate); at the end of the incubation and development with complement the plates were observed, and a cell dilution was chosen which would give between 10 and 100 anti-alanyl and anti-arsanil plaques per slide when plated with our modification of the Jerne assay. The original samples of immune cells were maintained in test tubes on ice throughout the period of this preliminary incubation and counting. The appropriate dilution was then assayed on microscope slides with $1.25 \cdot 10^6$ SRBC, pAlaSRBC, or ArsSRBC per slide and with $1.25 \cdot 10^6$ pAlaSRBC plus $1.25 \cdot 10^6$ ArsSRBC per slide, the latter being the "double" slides. In each case, six replicate slides were made with each single type of RBC sample, and between 40 and 85 replicates were made of the "double" slides.

Criteria for "double" plaques: A plaque was considered a "double" plaque if all the RBC within it (in the "double" slides) were lysed, and we were able to identify a single central PFC.

RESULTS

The first series of experiments was designed to test whether immunization with two antigenically non–cross-reacting erythrocytes will produce cells which simultaneously form antibodies of two distinct specificities. Preliminary experiments showed no observable cross-reaction between camel and rabbit RBC, nor between sheep and chicken RBC. In both antigen pairs, we were able to identify RBC as to their species of origin by the morphology of the erythrocytes. Both camel and chicken RBC are oval, whereas sheep and rabbit RBC are round. In addition, the chicken RBC are nucleated. Upon actual plating of immune spleen cells with these two antigen pairs, we found the chicken erythrocytes unsuitable for use in a search for clean "double" plaques, because their nuclei do not lyse upon immune hemolysis of the cytoplasmic membrane. Even though these nuclei are smaller than the rabbit erythrocyte, the presence of such nuclear bodies within an otherwise clean plaque might interfere with the detection of doubly active cells. We, therefore, performed this series of experiments with the camel-rabbit RBC pair exclusively. Thus, one group of mice was immunized with rabbit RBC (one intravenous inoculation of $4 \cdot 10^8$ RBC in 0.4 ml saline), the second group was similarly immunized with camel RBC, and a third was immunized with a single intravenous inoculum containing both rabbit and camel RBC. 4 days later, the animals were sacrificed and the spleen cells tested for single and double plaque formers. The results of this series of experiments are summarized in Table I. They indicate that among the 6000 anti-rabbit

plaques and the 11,000 anti-camel plaques counted, we were unable to detect a plaque in which the camel and the rabbit RBC had both been lysed.

One may argue that an immune competent cell can produce antibodies of two distinct specificities if, and only if, it is stimulated *simultaneously* by the two antigenic determinants. The antigenic determinants leading to antibody formation in the above series of experiments were carried by separate erythrocytes, and since the chances that two such RBC will stimulate a single competent cell simultaneously may be infinitely small, the incidence of doubles was zero. Therefore, the following experiments were performed with a single carrier molecule on which two non–cross-reacting antigenic determinants were chemically coupled.

TABLE I

Response of C3H Female Mice to Camel and Rabbit RBC: the Frequency of Single and "Double" Plaques

Immunization with:	Total No. of mice	Total anti-rabbit plaques	Total anti-camel plaques	Average No. of anti-rabbit PFC per 10^6 spleen cells	Average No. of anti-camel PFC per 10^6 spleen cells	Total No. of cells plated ($\times 10^6$)	No. of "double" plaques
4×10^8 rabbit RBC	16	4,315	20	465*	2.6*	7.24*	0
4×10^8 camel RBC	16	16	7,447	1.2*	695*	10.06*	0
4×10^8 rabbit RBC plus 4×10^8 camel RBC	26	6,166	10,738	340‡	629‡	15.11‡	0

* Computation based on results from 12 out of 16 mice on which complete cell counts were performed.

‡ Computation based on 20 out of 26 mice on which complete cell counts were performed.

The two determinants were peptides of DL-alanine, and *p*-azobenzenearsonate groups. Both determinants were attached to the same molecules of HSA leading to a modified protein preparation denoted pAlaArsHSA. For this series of experiments we worked with rabbits, since we were unable to effectively immunize mice to haptens coupled to HSA.

In a preliminary experiment with arsanil HSA, prepared similarly to antigen 1029, we determined that our immunization procedure induces a major response to the hapten in the draining lymph nodes and not in the spleen. Four rabbits were immunized intradermally with 20 mg of ArsHSA in complete Freund's adjuvant. On days 5, 6, 8, and 13 after immunization a rabbit was sacrificed, and aliquots of spleen and pooled lymph node cells were plated separately with ArsSRBC and SRBC. The results of this experiment are plotted in Fig. 1. We, therefore, performed all subsequent experiments on lymph nodes taken 8 days after immunization.

Six rabbits were immunized with antigen 1029 and five rabbits with antigen 1041. Tables II and III represent the data obtained from these two groups of animals. The results were computed by determining the number of PFC per

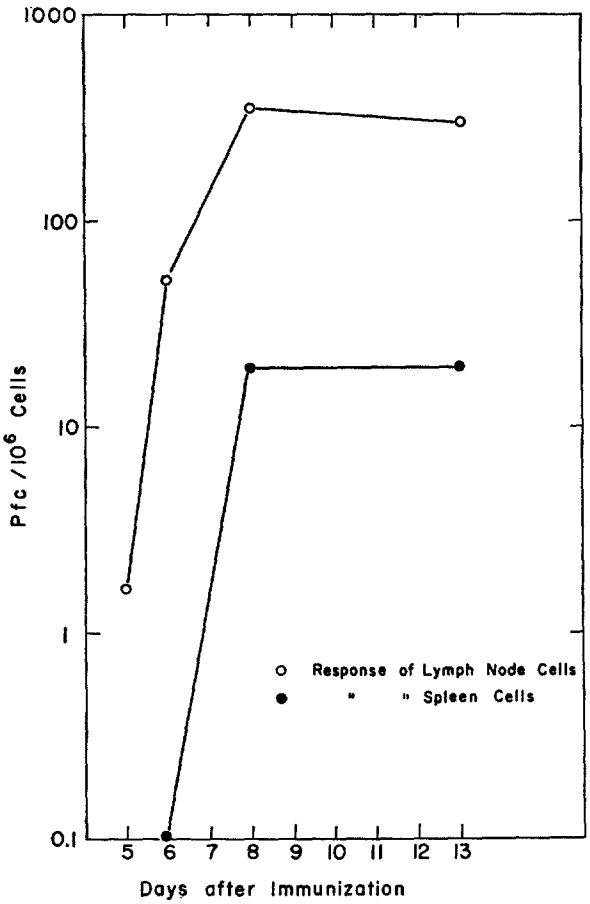

FIG. 1. Response of rabbit lymph node and spleen cells to multiple site, intradermal immunization with ArsHSA in complete Freund's adjuvant. Each point has been corrected for natural anti-SRBC PFC by subtracting the number of PFC/10^6 cells obtained against SRBC alone from that obtained with the conjugated ArsSRBC.

10^6 lymph node cells which responded to each hapten-coupled erythrocyte and to SRBC when plated individually. These results were then used to compute the number of single type anti-hapten plaques developed in the 40–85 replicate "double" slides as well as the expected number of anti-SRBC plaques in the "double" slides. Since the hapten-coupled SRBC are sensitive to lysis by anti-

sera to SRBC as well as antisera to the specific hapten coupled to the SRBC, the natural antibody plaques against SRBC register as clean "double" plaques in our assay. The number of expected natural anti-SRBC plaques was, therefore, computed and used as the base line in our search for cells producing antibodies of two specificities. As can be seen in Tables II and III, we were unable to detect

TABLE II

Response to Antigen 1029: the Frequency of Single and "Double" Plaques

Rabbit No.	Total No. of lymph node cells plated ($\times 10^6$)	Total anti-alanyl plaques	Total anti-arsanil plaques	Anti-SRBC PFC/10^6 lymph node cells	"Double" plaques	
					Expected anti-SRBC	Observed "double" plaques
142	1.23	192	644	0.54	0.66	2
143	1.6	560	2630	0.75	1.2	1
144	0.63	53	1730	0.93	0.58	0
148	4.37	238	1206	0.32	1.4	0
160	8.1	944	390	0.10	0.81	0
170	13.1	1200	1100	0.11	1.4	0
Total	29.03	3187	7700		6.05	3

TABLE III

Response to Antigen 1041: the Frequency of Single and "Double" Plaques

Rabbit No.	Total No. of lymph node cells plated ($\times 10^6$)	Total anti-alanyl plaques	Total anti-arsanil plaques	Anti-SRBC PFC/10^6 lymph node cells	"Double" plaques	
					Expected anti-SRBC	Observed "double" plaques
157	35.7	4270	1660	0.2	7.3	6
161	15.8	824	525	0.33	5.25	5
162	27.7	1415	1888	0.7	19.4	20
171	16.6	1298	2800	0.34	5.7	4
172	8.65	1139	1139	0.23	1.96	0
Total	104.45	8946	8102		39.61	35

any "double" antibody-producing cells above the expected background of anti-SRBC plaques.

DISCUSSION

The experiments reported in the present study on the production of antibodies by single cells indicated that after immunization of an animal with two different antigens or with two distinct antigenic determinants attached to one

molecule, each cell produced antibody of only one specificity. Similar observations have been made in previous studies (13, 15, 16),[1] yet, other reports claimed to have demonstrated the production of antibodies of two specificities by single cells (9–11, 14).

A search for single cells simultaneously producing antibody to two randomly chosen antigens requires, by the nature of the rarity or conceivable nonexistence of such cells, the screening of very large populations of lymphoid cells. The hemolysin plaque assay (17) enables one to study such large numbers of cells. In this present study we found it necessary to alter the original method of Jerne et al. in order that the bed of agar in which the plaques were situated be no thicker than the diameter of a plaque. This modification ensured the observation of plaques unobscured by a layer or layers of unlysed erythrocytes either above or below the plane of the plaque and facilitated the search for "double" plaques, clean of erythrocytes, in a background containing two antigenic types.

Friedman (16) used an unmodified Jerne plaque assay to study the frequency of mouse spleen cells simultaneously producing antibody to chicken and sheep RBC. In our hands, none of the plaques of lysed chicken RBC were clean because of the resistance of the nucleus of the chicken erythrocyte to lyses with anti-RBC hemolysins. Such a residue of nuclei might interfere with the detection of plaques clear of the two types of RBC. We, therefore, discarded the chicken-sheep RBC system and concentrated our efforts on a study of the camel-rabbit RBC pair. As can be seen from Table I, we were unable to detect "double" antibody-producing cells in a population of over $1.5 \cdot 10^7$ spleen cells, from 26 mice, which produced 6166 anti-rabbit RBC plaques and 10,738 anti-camel RBC plaques. The plaques observed against camel RBC in the mice immunized with rabbit RBC, and vice versa, represent natural anti-RBC plaques, detected even in normal unimmunized mice (17, 26, 27, 28), and do not reflect cross-reaction between the antigens tested.

In considering the possibility that one cell may simultaneously respond to two antigenic determinants, it would appear that the chances of observing such "double" antibody producers, if they exist, might be enhanced if the two determinants were so linked that they traveled together as a single molecule. Thus, any cell stimulated by one of the antigenic determinants would have a much greater chance for contact and potential stimulation with the second determinant. This approach has in fact been applied by Green et al. (15). Their results, published during the course of this study, were identical with ours, albeit based on a fluorescent antibody assay of cells from animals which had been immunized with conjugates of 2,4-dinitrophenyl hapten with bovine serum albumin or bovine gamma globulin. This antigenic system was therefore composed of one defined determinant—the hapten, and a variety of unspecified carrier determinants. In the present studies we chose to use an artificial antigen

composed of a protein carrier (HSA) onto which two chemically defined and non–cross-reacting antigenic determinants were attached ("arsanil" and polyalanyl groups). Two conjugates which differed in both absolute and relative amounts of "arsanil" and polyalanyl groups were used. It was felt that the study of two such antigens would lessen the possibility that greater immunopotency of one of the haptens might mask a potential response to the second hapten. We also felt that the search for double producers based on the evaluation of fluorescent dyes (15), might pose some difficulties in the subjective determinations. Our assay for anti-hapten antibodies entailed coupling the individual haptens to SRBC. Each rabbit tested demonstrated natural background plaques to SRBC. This background of natural PFC to SRBC was the base line in our assay for "double" antibody-producing cells. The presence of these natural antibody plaques provided us with an internal control of our system, since they allowed us to confirm that we can truly obtain clean plaques when both types of RBC, regardless of the hapten coupled to them, are sensitive to the antibody released by a single PFC. We were unable to detect "double" plaques in numbers greater than the expected amount of anti-SRBC natural plaques.

It should also be noted that the results indicating that no cells producing antibody of two specificities were registered in our investigation are based on the testing of 44,749 reacting cells, as compared to 1312 cells in the study of Green et al. (15). Thus, the conclusion that at a given time a cell produces antibody of only one specificity seems to be well established.

In drawing this conclusion from the results presented in this paper, we must however bear in mind the limitations of our assay. The Jerne assay, as performed here, detects only 19S hemolytic antibody which is released from the PFC in significant amounts. Thus, if a single cell were producing antibodies of two specificities one or both of which were not 19S hemolytic antibody, our assay would not register it as a "double" producer. Nor would we observe the "double" nature of a cell producing large amounts of antibody of one specificity and trace amounts of a second antibody, since such trace amounts might not suffice to produce a plaque of detectable diameter.

Some of the previous investigations claimed to have found a relatively high incidence of cells producing antibodies of two distinct specificities. Attardi et al. (11) claimed to have demonstrated cells neutralizing phages of two antigenic specificities. These claims were not confirmed by Mäkelä,[1] who demonstrated, in a series of strictly controlled experiments, using T2 and T5 phages as immunogens, that doubly active cells were not found. Hiramoto and Hamlin (14) reported that 45% of the cells which produced anti-human IgG globulin from guinea pigs which had been immunized with this antigen, produced both anti-Fc and anti-$F(ab')_2$. However, a detailed experimental analysis of this system, performed by Green et al. (15), demonstrated that the results of Hiramoto and Hamlin may be attributed to contamination of the antigenic reagents, probably

due to impurity of the Fc fragments. It is evident that the antigenic heterogeneity of the human IgG globulin makes this antigen very unsuitable for the analysis of antibody specificity produced by single cells.

We therefore conclude that at least for 19S antibody there must be some mechanism which prevents a cell from synthesizing antibody of more than one specificity at a time. To determine whether this is attributable to a limited amount of information in the genome or some other regulatory mechanism requires further experimentation.

SUMMARY

Two series of experiments were performed, utilizing a modification of the hemolysin plaque technique which registers 19S antibody, in an attempt to determine the frequency of cells capable of simultaneously producing antibody to two non–cross-reacting antigens. Mice were immunized i.v. with rabbit and camel RBC and their spleens assayed for cells producing antibody against both antigens. 16,904 cells producing antibody of one or the other specificity, from 26 mice, were counted. Not one cell was detected which produced antibody of two specificities. Rabbits were immunized intradermally with HSA to which polyalanyl and *p*-azobenzenearsonate groups were chemically attached. The individual haptens, polyalanyl, and *p*-azobenzenearsonate groups were coupled to separate aliquots of SRBC, and the lymph nodes of immunized rabbits were assayed for cells releasing antibody against both haptens. In a study of 11 rabbits, after counting 27,845 cells producing antibody, we detected no "double" plaques.

This work was supported by a Grant No. C-6165 from the National Institutes of Health, United States Public Health Service, Bethesda, and by the Air Force Office of Scientific Research through the European Office of Aerospace Research, U.S. Air Force, under grant 67-19.

BIBLIOGRAPHY

1. Burnet, F. M. 1959. The Clonal Selection Theory of Acquired Immunity. Vanderbilt Univ. Press, Nashville, Tenn.
2. Cohen, S., and C. Milstein. 1957. Structure of antibody molecules. *Nature.* **214**:449.
3. Edelman, G. M., and J. A. Gally. 1967. Somatic recombination of duplicated genes: an hypothesis on the origin of antibody diversity. *Proc. Natl. Acad. Sci. U.S.* **57**:353.
4. Smithies, O. 1967. Antibody variability. *Science.* **157**:267.
5. Lederberg, J. 1959. Genes and antibodies. *Science.* **129**:1649.
6. Szilard, L. 1960. The molecular basis of antibody formation. *Proc. Natl. Acad. Sci. U.S.* **46**:293.
7. Dreyer, W. J., and J. C. Bennet. 1965. The molecular basis of antibody formation: a paradox. *Proc. Natl. Acad. Sci. U.S.* **54**:864.
8. Haurowitz, F. 1965. Antibody formation and the coding problem. *Nature.* **205**:847.
9. Nossal, G. J. V., and O. Mäkelä. 1962. Kinetic studies on the incidence of cells appearing to form two antibodies. *J. Immunol.* **88**:604.

10. Schwartzman, J. S. 1967. Effect of the number of antigens on the quantity of cells producing several kinds of antibody. *Nature.* **213:**925.
11. Attardi, G., M. Cohn, K. Horibata, and M. Lennox. 1964. Antibody formation of rabbit lymph node cells. I. Single cell responses to several antigens. *J. Immunol.* **92:**335.
12. White, R. G. 1958. Antibody production by single cells. *Nature.* **182:**1383.
13. Vasquez, J. J. 1961. Antibody or gamma globulin forming cells, as observed by the fluorescent antibody technique. *Lab. Invest.* **10:**1110.
14. Hiramoto, R. N., and M. Hamlin. 1965. Detection of two antibodies in a single plasma cell by the paired fluorescence technique. *J. Immunol.* **95:**214.
15. Green, I., P. Vassalli, V. Nussenzweig, and B. Benacerraf. 1967. Specificity of the antibodies produced by single cells following immunization with antigens bearing two types of antigenic determinants. *J. Exptl. Med.* **125:**511.
16. Friedman, H. 1964. Monospecific antibody plaque formation by spleen cells immunized with sheep and chicken erythrocytes. *Experientia.* **20:**564.
17. Jerne, N., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody producing cells. *In* Conference on Cell Bound Antibodies. B. Amos and H. Koprowski, editors. The Wistar Inst. Press, Philadelphia. 109.
18. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. Charles C. Thomas, Springfield. 149.
19. Tabachnick, M., and H. Sobotka. 1959. Spectrophotometric studies of amino acid azo derivatives. *J. Biol. Chem.* **234:**1726.
20. Schechter, I., S. Bauminger, M. Sela, D. Nachtigal, and M. Feldman. 1964. Immune response to polypeptidyl proteins in rabbits tolerant to the protein carriers. *Immunochemistry.* **1:**243.
21. Sela, M., and A. Berger. 1955. The terminal groups of poly-α-amino acids. *J. Am. Chem. Soc.* **77:**1893.
22. Tabachnick, M., and H. Sobotka. 1960. Azoproteins: a spectrophotometric study of the coupling of diazotized arsanilic acid with proteins. *J. Biol. Chem.* **235:**1051.
23. Anfinsen, C. B., M. Sela, and J. P. Cooke. 1962. The reversible reduction of disulfide bonds in polyalanyl ribonuclease. *J. Biol. Chem.* **237:**1825.
24. Ingraham, J. S. 1952. Specific complement dependent hemolysis of sheep erythrocytes by antiserum to azo hapten groups. *J. Infect. Diseases.* **91:**268.
25. Rimon, A., and M. Sela. 1966. Chemical modification of human erythrocytes by attachment of tyrosine and alanine peptides. *Biochim. Biophys. Acta.* **124:**408.
26. Cunningham, A. J. 1965. A method of increased sensitivity for detecting single antibody forming cells. *Nature.* **207:**1106.
27. Friedman, H. 1964. Distribution of antibody plaque forming cells in various tissues of several strains of mice injected with sheep erythrocytes. *Proc. Soc. Exptl. Biol. Med.* **117:**526.
28. Hege, J. S., and L. J. Cole. 1967. Antibody plaque forming cells in unsensitized mice. *J. Immunol.* **99:**61.