Antibody-Forming Cells: Population Patterns after Simultaneous Immunization with Different Isoantigens

Abstract. *Spleen cells were obtained from chickens stimulated simultaneously with different erythrocytic antigens, and the proportions producing one and two antibodies were determined by the hemolytic-plaque technique. Most cells from birds immunized with two antigens of the same system (B) appeared to produce only one antibody. In a population of cells from birds immunized with antigens of different systems (A and B), a relatively high proportion of cells produced two antibodies.*

Lymphoid cells producing hemolytic antibody continue to liberate the antibody when mixed with target erythrocytes in solidified agar. Zones of hemolysis (plaques) are produced, each of which represents the effect of antibody released by a single cell (*1*). Since this method permits the study of large numbers of antibody-forming cells, we have used it to determine whether populations of lymphoid cells from animals stimulated simultaneously with two isoantigens produce and release antibodies of single or double specificity. If all cells form antibodies to both antigens, an equal number of plaques should be formed in agar containing target erythrocytes having either both or only one of the two foreign antigens. But if all cells produce antibodies of a single specificity, the number of plaques formed with target erythrocytes having both antigens should equal the sum of the numbers of plaques formed when the two antigens are tested separately. Values intermediate between these two extremes would indicate that some cells produce two antibodies with different specificities and others produce antibodies having a single specificity (*2*).

Young adult chickens from a moderately inbred line (inbreeding coefficient approximately equal to 0.60) were typed for five blood-group systems (A, B, C, D, and L) and used in two separate experiments. Isoimmunization and blood-group studies in the last eight generations suggest that this line is genetically homogeneous for blood group systems other than those mentioned above. In experiment 1, five birds were immunized with erythrocytes from a single donor that had only foreign antigens of the B blood-group system. Genotypes of donor and recipients were B^2/B^3 and B^1/B^1, respectively. From 3 to 8 days after the animals had been given two immunizing injections, the spleen cells of each were tested separately with five types of erythrocytes. These were B^2/B^3, B^1/B^2, B^1/B^3, B^2/B^2, and B^3/B^3. In experiment 2, six birds (A^1/A^1, B^1/B^1 genotype) were similarly immunized with erythrocytes from a single donor having only foreign antigens of A and B blood groups (A^1/A^2, B^2/B^3 genotype) (*3*). Spleen cells were tested separately with three types of erythrocytes: donor type, the type having foreign A antigen (A^1/A^2, B^1/B^1 genotype), and the type having foreign B antigen (A^1/A^1, B^2/B^3 genotype). Although three foreign antigens (A_2, B_2, B_3) were involved in this experiment, the B antigens were treated as a single antigen in the plaque tests. This did not prevent our determining whether cells producing antibody to A produce one or two antibodies. The frequency that erythrocytes from birds of the B^2/B^3 genotype have only B_2 or B_3 antigens (allelic exclusion phenomenon) is extremely low or possibly zero. Agglutination tests with strong antiserums also indicate that probably all erythrocytes from chickens with A^1/A^2, B^2/B^3 genotypes have A_2 antigens.

The B_2 and B_3 antigens controlled by the same system represent complex antigens with some common factors or specificities. The possibility of antibodies cross-reacting with A and B antigens is remote since these antigens are determined by genes at different loci and display independent inheritance.

The method used in the hemolytic-plaque tests was similar to that described by Jerne and Nordin (*1*). A sample of viable cells from the entire spleen (2×10^6 cells not stained by trypan blue) was tested in duplicate with each type of target erythrocyte. Although chicken isoantibodies are not hemolytic, plaque formation was obtained when rabbit antiserum to chicken globulin was added along with guinea pig complement after an initial incubation of spleen cells with target erythrocytes for 1.5 hours.

After the second incubation period of 1 hour, the plaques were counted (3× magnification) with dark-field illumination. The results were expressed as the mean number of plaques per unit area of the duplicate determinations.

Probably the antibody to the globulin caused plaque formation regardless of the molecular weight of the isoantibodies (*4*). Differences in hemolytic efficiency, as related to the molecular weight of the isoantibodies, are probably not important in our study. That spleen cells from normal birds (unimmunized) do not produce plaques in this system suggests that the initial isoimmunization produces a primary immune response.

An analysis of variance for each experiment, based on the mean number of plaques per unit area, showed a highly significant difference ($P < .01$) attributable to the type of target erythrocyte (Tables 1 and 2). To simplify comparisons, the plaque counts for each erythrocyte type are expressed as the percentage of those obtained with erythrocytes of the donor type. The latter were arbitrarily assigned the number 100.0.

At least some spleen cells formed antibodies having a single specificity (Table 1). In all cases the number of plaques formed with B^2/B^3 erythrocytes was greater than the number formed with any other type of erythrocyte. However, an exact estimate of the proportions of cells producing one or two antibodies is difficult because of the significant antigen-dosage effect (compare columns b with d and c

Table 1. Relative percentage of plaque-forming cells from B^1/B^1 birds immunized with erythrocytes from a B^2/B^3 donor.

Spleen (bird number)	Target erythrocyte type						
	(a) B^2/B^3	(b) B^1/B^2	(c) B^1/B^3	(b+c)	(d) B^2/B^2	(e) B^3/B^3	(d+e)
145	100.0	30.4	77.1	107.5	68.0	90.3	158.3
146	100.0	65.7	47.2	112.9	82.9	52.9	135.8
147	100.0	43.3	65.1	108.4	51.7	71.2	122.9
151	100.0	20.3	38.8	59.1	43.7	63.2	106.9
152	100.0	38.5	71.6	110.1	42.0	92.4	134.4

with e). It is not clear why twice as much antigen on the erythrocytes should increase the number of plaques. The fact that it did suggests that plaque counts in tests with B^1/B^2 and B^1/B^3 erythrocytes are low estimates of the true number of cells forming antibodies to antigens B_2 and B_3. This may be due to subthreshold quantities of antibodies, formed by some spleen cells, which are insufficient to sensitize B^1/B^2 and B^1/B^3 erythrocytes adequately for subsequent lysis by complement and antibody to globulin. This amount of antibody would be adequate for lysis of the homozygous type erythrocytes possibly because of a difference in the local concentration of the foreign B antigens on the cell surface.

If all cells produced and released the same amounts of antibodies to both foreign antigens, equal numbers of plaques should have been found in tests with B^2/B^3, B^2/B^2, and B^3/B^3 erythrocytes because the total antigen dosage was essentially equivalent among the three erythrocyte types. This was not the case (Table 1, columns a, d, and e). Conversely, if there were two separate populations of cells, each of which produced only antibodies to B_2 or B_3 antigens, the number of plaques found in tests with B^2/B^3 erythrocytes should have approximated the sum of the plaque counts obtained in tests with B^1/B^2 and B^1/B^3 erythrocytes. In this situation the three types of erythrocytes have a single dose of the relevant antigens. Here the only antigen-dosage effect possible, with respect to B^2/B^3 erythrocytes, would be that due to a double quantity of antigenic factors shared by B_2 and B_3 antigens *(5)*. The data in Table 1 (columns a, b, and c) closely fit this model of two distinct populations of cells each producing antibodies to either B_2 or B_3 antigens. The only other conclusion possible is that there were three populations of cells: cells producing antibodies to B_2 antigen, those producing antibodies to B_3 antigens, and cells producing both. Results obtained with four of the spleen cell samples suggest that the number of cells which produced both antibodies was relatively low. Conclusions cannot be drawn from the plaque counts with cells from bird No. 151 in view of unexplained low numbers of plaques formed in tests with B^1/B^2 and B^1/B^3 type erythrocytes.

A comparison of the means of the data from which Table 2 is drawn showed that plaque counts in tests with target erythrocytes having both B and A antigens do not differ significantly from those obtained with erythrocytes having B antigens alone. Since an average of 29.6 percent of the cells which reacted with erythrocytes having both B and A antigens also reacted with erythrocytes having only A, we conclude that cells which produce antibodies to A are members of the population producing antibody to B. These data, with but two possible exceptions (that from birds 385 and 450), imply that all cells which produce antibodies to A also produce antibodies to B. Because erythrocytes from birds of appropriate genotypes were unavailable, it could not be determined whether the cells which formed antibodies to A also formed antibodies to B_2 or B_3, or both.

Table 2. Relative percentage of plaque-forming cells from birds immunized with erythrocytes having foreign B and A antigens.

Spleen (bird number)	Antigen(s) of target erythrocytes		
	B + A	B	A
385	100.0	105.5*	9.0
387	100.0	103.4*	35.0
388	100.0	102.0*	33.1
448	100.0	95.9	36.1
449	100.0	93.2	60.2
450	100.0	91.4	4.1

* Spleen cells from three birds produced more plaques against B than B + A and hence the relative percentage in each instance exceeds 100. This discrepancy is attributable to sampling error.

The results of experiments 1 and 2 indicate that different patterns of antibody-forming cells arise in response to two different combinations of isoantigens. The ability to demonstrate that cells may produce one or two antibodies may simply depend on the choice of antigens. This may account for differences in incidence of cells producing two antibodies as reported by others *(6)*. That both antigens used for immunization were present on the same erythrocyte may have been relevant to the results of experiment 2.

In this case, if cells can produce two antibodies—provided the proper antigens "hit" the appropriate cell at approximately the same instant, there would have been a greater chance for "double-hits" than if the antigens had been on separate carriers or had been administered serially *(6)*. It may be significant that the best evidence that cells produce two antibodies appeared in response to antigens determined by different genetic loci, whereas a higher proportion of cells with specificity for a single antibody characterized the response to antigens controlled by the same system. The latter is in accord with the results of a study with chickens in which the graft-versus-host reaction was used as the assay system *(7)*. Here, too, lymphoid cells appeared to have single specificity to each of two B antigens. Whether or not there is a genetic mechanism that regulates the relative size of each population of antibody-producing cells is uncertain. However, the spleen from bird No. 146 contained considerably more cells producing antibody to B_2 than to B_3, a relation opposite to that found with the other four birds (Table 1).

One interpretation of the results of experiment 1 fits a clonal selection hypothesis as originally postulated *(8)*. On the other hand, the finding that a large percentage of cells may produce two antibodies (experiment 2) is incompatible with a strict selectionist theory in which frequent random mutation accounts for diverse populations of cells each with limited specificities. If mutation is frequent and random for a single locus, double-antibody producing cells might exist, but the number of cells producing antibodies against any two antigens should be very low *(9)*.

On the basis of a gene-loss mechanism, to account for a diversity of antibody-controlling loci, one would expect to find more cells producing two antibodies in experiment 1 than in experiment 2, since according to this theory *(10)* fewer losses occur for the strong antigens (such as B) than for weaker antigens (such as A). The results, though not conclusive, do not reflect this trend.

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References and Notes

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2. We assume that variations in plaque counts with different erythrocyte types are not due to differences in lysability. The significance of different isoantigens to the structural integrity of the chicken erythrocyte is unknown.
3. The letter designation for the A and B blood group systems are specific and correspond to those used by other workers whereas the numerical superscripts, which designate specific alleles, apply only to the strain of chickens used in this study.
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of plaques was less than 5 percent of the number obtained in tests with B^3/B^3 erythrocytes.
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Isozymes of Aldolase

Abstract. *A new method of starch-gel electrophoresis using a technique of staining dependent on enzyme activity has been employed to demonstrate the isozymes of aldolase from a variety of human, rat, and frog tissues. Five of these isozymes were detected in man, seven in the rat, and at least four in the frog. The abilities of these isozymes to cleave fructose-1,6-diphosphate and fructose-1-phosphate were compared.*

Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphatelyase) catalyzes the cleavage of fructose-1,6-diphosphate (FDP) to triose phosphate. Since there are at least two forms of this enzyme, distinguished by their different abilities to split fructose-1-phosphate (*1–3*), we conducted experiments to determine the existence of aldolase isozymes. By modifying the enzymatic assay originally described by Sibley and Lehninger (*4*), we have developed the first method for demonstrating these isozymes with starch-gel electrophoresis. In principle, the triosephosphates formed by the action of aldolase upon FDP or F-1-P (fructose-1-phosphate) are trapped by hydrazine and detected by the colored product resulting from their reaction with 2,4-dinitrophenylhydrazine.

Tissue samples were obtained from Wistar rats and frogs (*Rana pipiens*), and from human cadavers no later than 12 hours post-mortem. Because the aldolase content varies from one tissue to another, we diluted the tissue homogenates accordingly; the dilutions ranged from 4 g (wet weight) of testis in 4 ml of disodium ethylenediaminetetraacetate (EDTA) to 0.2 g of skeletal muscle in 6 ml of EDTA. By using this technique, we obtained colored spots of similar intensities. Samples were homogenized in a modified Potter-Elvehjem homogenizer with cold 0.1*M* Na_2 EDTA, *p*H 7.5. The homogenates were centrifuged for 1 hour at 20,000*g* and stored at 4°C until they were introduced into the gel. A horizontal gel was prepared with Connaught hydrolyzed starch (106 percent of the recommend amount) and EBT buffer [0.02*M* EDTA, 0.5*M* boric acid, 0.9*M* tris(hydroxymethyl)aminomethane (tris)] at *p*H 8.6 (*5*). Electrophoresis was performed for 20 to 22 hours at 4°C at 3.0 to 4.0 volt/cm.

Reagents used in the staining procedure were: the substrates, 0.1*M* FDP or 0.1*M* F-1-P (sodium salts prepared from the barium salts obtained from Calbiochem) at *p*H 8.6; 0.56*M* hydrazine sulfate, *p*H 8.6; and tris-HCl buffer, *p*H 8.6. The sliced gels were incubated in a mixture of 15 ml of substrate, 5 ml of hydrazine, 50 ml of buffer, and 0.5 g of albumin for 45 minutes at 37°C. Incubation in a slowly shaking water bath reduced incubation time to 35 minutes. After incubation, the substrate was removed by aspiration. The gels were flooded with 2,4-dinitrophenylhydrazine in 0.5*M* HCl and were incubated again for 45 minutes at 37°C. The aldolase spots appeared after treatment of the gel surface with 0.6*N* NaOH for approximately 10 minutes. These spots were red on a yellow background; hence, photographing them was somewhat difficult. The red color was transitory, and the NaOH rapidly hydrolyzed the gel surfaces; preservation was enhanced by the removal of the NaOH

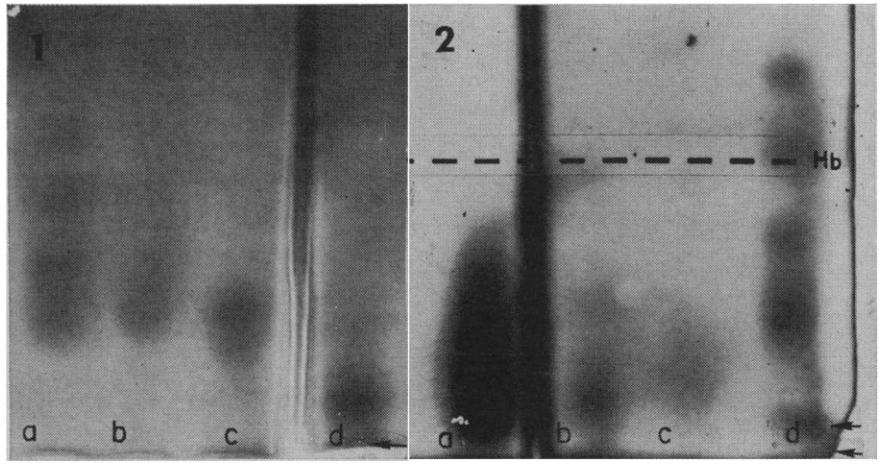

Fig. 1. Isozymes from human cadavers. *a*, Brain; *b*, heart; *c*, rabbit muscle aldolase, control (Boehringer and Soehne, concentration about .008 International Enzyme Units); *d*, human liver. Electrophoresis for 22 hours at 3 volt/cm. The arrow indicates the origin. Fig. 2. Patterns of aldolase from human tissue. *a*, Muscle; *b*, liver; *c*, kidney; *d*, brain. Five isozymes are distinguishable. Mark nearest origin of *d* is an artifact caused by destruction of the gel by NaOH. Hemoglobin (*Hb*) shows as a faint bar in muscle, liver, and kidney tracks. Electrophoresis was carried out for 22 hours at 4 volt/cm. The top arrow points to the artifact; the bottom, to the origin.

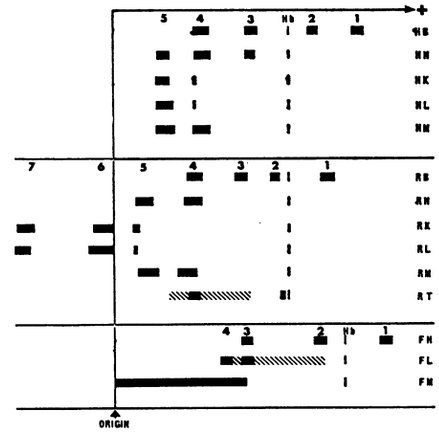

Fig. 3. Scale representation of relative mobilities of aldolase isozymes. Top to bottom: human brain, heart, kidney, liver, and muscle; rat brain, heart, kidney, liver, muscle, and testis; and frog heart, liver, and muscle. Various lengths of bands are probably indicative of quantity of each isozyme. Hb represents hemoglobin bands. (Direction of electrophoretic migration is from origin to right.)