THE FREQUENCY OF ANTIGEN-SENSITIVE CELLS IN TISSUE TRANSPLANTATION

A Commentary on Clonal Selection

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(Received for publication 21 October 1968)

One of the obvious ways of testing the clonal selection hypothesis is to determine the minimum number of cells needed to initiate a measurable immune response. At least in the uncompromising, and now classical, hypothesis of one antigen-sensitive cell clone per antibody specificity (1, 2) it is mandatory that a given antigenic determinant should not be recognizable by every small lymphocyte—accepting that antigen-sensitive cells are to be found among this, in several ways, heterogenous cell population. While this extreme result, if found, would truly invalidate the hypothesis it is unfortunately not possible to define exactly the maximum frequency of antigen-sensitive cells which would still be compatible with the hypothesis. Clearly, the more complex the immunological universe is thought to be, the higher becomes the number of different clones required by the hypothesis, and the lower becomes the expected frequency of a cell belonging to one particular clone. Few immunologists would probably expect a frequency higher than 10^{-4}.

For reasons which were suggested by earlier studies of the factor of immunization (3), the strong histocompatibility antigens were considered to provide a particularly exacting test of the clonal selection hypothesis. The graft-vs.-host (GVH) reactions in chicken embryos injected intravenously with adult chicken lymphocytes were used in the present study to determine the frequency of antigen-sensitive cells with respect to the strong antigens of the B locus (4). It is concluded that their frequency is too high to be compatible with the orthodox version of clonal selection.

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Material and Methods

Experimental Procedure.—Blood lymphocytes from adult cocks of known homozygocity for the B locus were injected intravenously into 10-day heterozygous embryos which shared one B allele with the donor. The recipients were killed 11 days later (the day of hatching) and were scored for macroscopic signs of GVH reaction by their spleen weights. The spleens of female recipients were examined cytologically for metaphases of donor origin as described below.

The blood to be injected was freshly drawn from a vein in one part of 3.8% sodium citrate to nine parts of blood and was diluted before injection in citrated phosphate-buffered saline (PBS). The lymphocyte counts in adult cocks are of the order of 10,000/μl. Thus dilutions of 1:10,000 contained roughly 50 lymphocytes/0.05 ml administered intravenously with a glass tuberculin syringe and a 30 gauge needle. Attempts at fractionating the blood elements were deliberately avoided in order to minimize cell damage and/or clumping. Each experiment contained a group of spleen weight controls injected with citrated PBS alone.

Chickens.—The B blood group chickens were of stock which was formerly bred by Thornber Brothers (Halifax, Yorkshire, England), but this flock is now established at the Charles Salt Research Centre, The Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, England, and were supplied to us by the courtesy of Mr. Michael McDermid.

Two B group combinations were used: (*a*) B 14/14 → B 2/14, and (*b*) B 19/19 → B 2/19. Control series consisted of B 19/19, B 2/2, or B 2/19 adult blood preparations injected into embryos of the same genotype as the donor (syngeneic with respect to the B locus but different in an unknown number of weaker loci). An additional series consisted of B 19/19 blood injected into outbred White Leghorn (WL) embryos of commercial closed breeding stock.

Chromosome Preparations.—Chromosome preparations were made as previously described with slight local modifications (5). Adequate preparations stained with 2% propionic orcein (6) and mounted permanently allow certain recognition of the female sex chromosome constitution ZW as distinct from the male ZZ constitution (7). Some slides were stained instead with Giemsa solution.

Several slides were made from each preparation and all readable metaphases on each slide were scored.

RESULTS

The data of Table I summarize a preliminary experiment set up to define the order of magnitude of the cell dose needed to induce splenomegaly in the two main stain combinations employed. It is obvious that 10,000 lymphocytes caused a massive splenomegaly in either combination, and also that 1000 cells were highly active in the B 14/14 → B 2/14 combination. In this combination chromosome analysis detected the occasional donor metaphase even in female recipients of 100 cells, although this dose gave no significant spleen enlargement.

In the main experiment with these combinations (Table II) it was thereafter decided to employ the doses of 5 and 50 cells by injection of B 14/14 → B 2/14, and the doses of 50 and 500 cells in the B 19/19 → B 2/19 combination. Furthermore, the timing was modified so that the embryos were injected at the 10th day instead of the 13th day of Table I, but they were still killed at the day of hatching. (Only group 3 of Table II which comprises some eggs from group 3 of Table I was injected at day 13.)

The material contained in Table II can be considered first as falling into

two main classes defined by the presence (groups 1–6) or absence (groups 7–10) of a B group stimulus to the potential GVH reaction. It is clear that the additional presence of a foreign B allele in the host reduces the number of donor cells needed to give a comparable frequency of response by a factor of

TABLE I

Spleen Enlargement Produced by Dilutions of Adult B Group Homozygous Donor Blood Injected Intravenously into Heterozygous 13-Day Embryos Killed at Day 21

No. of injected lymphocytes	B group combination	Spleen weights				
				mg		
10,000	14/14—2/14	104	162	190		
1,000	14/14—2/14	13	23	34	57	
100	14/14—2/14	8	12	13	18	19
10,000	19/19—2/19	27	48	50	64	203
1,000	19/19—2/19	12	12	13	15	16
100	19/19—2/19	5	12	12	12	15
0	controls	14 ± 2 (SD)				

TABLE II

Mitotic Donor Cell Response and Frequency of Spleen Enlargement Found in Embryos Injected Intravenously with Adult Male Chicken Blood Lymphocytes at Day 10 and Killed at Day 21

Group	Strain combination	No. of injected lympho-cytes	Chromosome analysis in ♀ recipients			Frequency of splenomegaly in both sexes together‡
			Donor metaphases		Frequency of mitotic responders*	
			Total read			
				%		
1	B 14/14 → B 2/14	5	0/16	0	0	0/17
2	"	50	23/404	5.7	3/6	6/40
3§	"	100	18/404	4.5	2/3	0/19
4	B 19/19 → B 2/19	50	13/546	2.4	4/7	1/75
5	"	500	23/569	4.0	2/6	8/43
6	B 19/19 → WL	500	15/1050	1.4	4/9	9/38
7		50	0/295	0	0	0/20
8	B group syngeneic	500	0/62	0	0	0/9
9	combinations	5,000	3/377	0.8	1/1	0/15
10		500,000	38/357	10.8	4/4	1/9

* Signifies the frequency with which one or more donor metaphases has been diagnosed in spleens where the readable number of total metaphases was sufficient to give an expected average of at least one (e.g. if the pooled readings of a group gave 4% donor metaphases individual spleen preparations are listed as nonresponders only if a minimum of 25 metaphases were readable and no donor metaphases found).

‡ Signifies the frequency with which the spleen weight exceeded 20 mg, i.e. the mean + 3 × SD. Most of the enlarged spleens weighed in fact several times the mean.

§ Group 3 was injected on the 13th instead of the 10th day.

10^2–10^3. This fact is, of course, in accordance with the view that antigenic strength merely reflects the frequency of the respective antigen-sensitive cells (3, 8).

Turning now to the B group incompatible combinations, the most important finding is the fact that a response is detectable at all with as few as 50–100 cells. Although the frequency of spleen enlargement is low, particularly so in group 4, the frequency of the mitotic donor response of 9/16 for groups 2–4 combined is really quite convincing. It shows that an inoculum of 50–100 cells is more likely than not to contain a minimum of one responder. The fact that the latter are indeed responding to the strong B group antigens is apparent from the comparison with the control groups 7–10. The observed frequency of antigen-sensitive cells of 1–2% of the lymphocyte population is, for reasons to be discussed, almost certainly a minimum estimate.

As a more trivial point it may be concluded from the last two vertical columns of Table II that karyological analysis of the spleen is a more sensitive test for GVH reaction than simple weighing. Even when female embryos alone are considered (Solomon [9] found female embryos to be more responsive than males), the frequency of embryos with donor metaphases but no spleen enlargement is higher than the reverse situation, which fact can hardly be surprising. Nor will anyone doubt that karyological examination is the more difficult and laborious method.

DISCUSSION

Before approaching the more controversial implications of our experiments it would be well to point out that there are several reasons why the frequency of 1–2% of responding lymphocytes to a single histocompatibility allele may be considered a minimum estimate.

1. Only a minority of the injected lymphocytes can be expected to settle in the spleen. On the other hand, cells which settle in a different site, as well as their mitotic descendants, may of course migrate to the spleen later.

2. Not all cells of the morphology of lymphocytes are actual or potential antibody producers. As Howard and his colleagues have shown (10), even small lymphocytes from the thoracic duct comprise members (of unknown frequency) which are precursors of macrophages.

3. Not every antigen-sensitive cell belonging to a given clone may be expected to take the road to immunity when meeting its predestined antigen. Some are known to respond instead by going which ever is the true way to tolerance, but their relative frequency is unknown.

4. Not every lymphocyte in the inoculum belonging to the right clone and responding by immunity, and doing so in the spleen, may be capable of continued proliferation over a minimum of 10 cycles, let alone of causing splenomegaly. No less than 10 consecutive divisions starting from a single cell would

be needed to give a final frequency of donor metaphases of 1%, and only few of our preparations permitted identification of more than 100 metaphases.

5. Also memory cells belong to the pool of circulating lymphocytes (11). The true frequency of antigen-sensitive cells would be underestimated if the number of memory cells were not subtracted from the inoculum. Failure to do so would tacitly imply that memory cells might be forgetful, i.e. liable to change their commitment, whether or not the latter is itself viewed as a clonal attribute. Unfortunately, we do not know how many of the circulating lymphocytes are memory cells. They might well form the majority.

6. The antigenic stimulus from a single foreign allele of the B locus is unlikely to be caused by a single antigenic determinant. By analogy with the strong histocompatibility loci of mice and men, *H-2* and *HL-A*, the alleles are much more likely to be complex (12). Let us suppose that the effective antigenic stimulus in our experiments is in fact composed of 5–10 determinants. If the experiments of Klein (13) with one particular combination of congeneic strains of mice are generally valid, they would imply in clonal terms that strong histocompatibility antigens derive their apparent strength from the fact that they engage simultaneously a multiplicity of clones (singly the determinants are no stronger than the antigens of reputedly weak loci). So then, if this is true, a GVH reaction given by 50 cells would mean that a minimum of 5–10 cells were initiating the response rather than a minimum of one. The frequency of each of the 5–10 clones could of course remain at the 1:50 level. But the combined fraction of antigen-sensitive cells responsive to a single histocompatibility allelle would rise to 10–20% even without allowing for underestimation of this fraction on the accounts listed in points 1–5. Admittedly, the above mentioned generalization from Klein's experiments might not be valid. If, on the contrary, a single antigenic determinant of a B allele would suffice to elicit the response we are measuring, then the frequency of responders could be overestimated on this account.

It stands to reason that the present experiments, of which a preliminary account and discussion was given by Simonsen (14), should mainly be of concern to immunologists because of their bearing on the clonal selection hypothesis. As interpreted by the authors, the findings are just not compatible with the hypothesis of one clone of antigen-sensitive cells per receptor specificity. The sum of clones responsive to all the alleles of a single histocompatibility locus would probably preempt or even exceed the immunological resources, unless each "clone" can also respond to other antigens, in other words, is multipotential.

Experience shows that protagonists of the clonal selection hypothesis in its orthodox version have three cardinal means of reconciling the present findings with their theory:

(*a*) that the observed reaction is not really immunological, but more akin

to the type of cell to cell interaction which is based on structural differences of the cell surface and which is believed also to underpin "allogeneic inhibition";

(*b*) that although the GVH reaction is a true immune reaction, it is a secondary rather than a primary response which is elicited by antigens of a strong histocompatibility locus;

(*c*) that although the GVH reaction to strong histocompatibility antigens is a real primary immune response, it is a peculiar one for the reason that even antigen-sensitive cells with receptors of very low avidity (in fact clones "belonging" to different antigens) are stimulated to proliferation. The strength of these antigens does not therefore rest with the large size of their corresponding clone, but instead with their ability also to stimulate what is in fact the clones of other antigens.

These views are clearly not held simultaneously.

As to the first of these possibilities, there are at least two compelling arguments against it:

A. In conditions where GVH reactions are readily produced by grafting from parental donors to their F_1 hybrids with different strains, the reverse procedure leads to no reaction. This principle has long been known to hold true for GVH reactions, in chicken embryos as well as for other well-defined GVH reactions, and may still be regarded as the most satisfactory proof that a given effect is indeed initiated by an immune reaction in the grafted cells against some antigen of the host.

B. Pretreatment of the donor in such a way that is is rendered specifically tolerant of the host precludes the otherwise regular development of a GVH reaction. This important principle was first demonstrated for GVH reactions in chicken embryos in Burnet's laboratory (15). It seems to be as clear a proof as can be devised that the GVH reactions observed were not a nonimmunological cell contact phenomenon. We have not in the present study conducted control experiments of the type described under *A* and *B*, although the *A* type control, as far as splenomegaly goes, has been done earlier with similar chicken material with the expected negative result.

With respect to the second of the raised possibilities, that the GVH reaction to strong transplantation antigens may be a secondary response, we shall refer to an earlier discussion (14). Suffice it here to restate the point that if the sum of lymphocytes which can respond to the antigens determined by the alleles of a single histocompatibility locus approaches the whole lymphocyte population, then it is of little, if any, help to the clonal selection hypothesis to assume that most of the responding cells are in fact performing a secondary response. We would still need antigen-sensitive cells for the rest of the antigens, unless we are also asked to suppose that these are cross-reacting with one or other antigenic determinant of the admittedly complex system of strong histocompatibility which each species seems to have developed. The latter

seems rather an unlikely possibility, and is at least not supported by the finding that GVH reactions in germfree mice take an apparently normal course (16).

Finally, the third possibility mentioned before is made unlikely by the same tolerance experiment which disposed of the first possibility. Removal or inactivation of the clones with specific receptors for a given histocompatibility antigen should presumably not eliminate the multitude of other clones with the postulated accidental low avidity receptors for the same antigen.

Although we are thus unable to find a plausible way of reconciling the present findings with clonal selection in its orthodox version they are compatible with any modification of clonal concepts which can allow for the existence of multipotential antigen-sensitive cells (3, 14).

The basic concept in molecular immunology, that the genetic information needed for the specificity of immunoglobulin synthesis is laid down in the antigen-sensitive cells before contact with antigen, is quite unchallenged by our conclusion that each antigen-sensitive cell must be capable of producing several different receptor molecules. Further, there is no reason why the heterogeneity among multipotential antigen-sensitive cells should not be sufficient to account for the known heterogeneity among antibody-producing cells.

In addition to the genetic heterogeneity of antigen-sensitive cells which has to do with the problems of clonal selection, the whole issue is now being further complicated by the demonstration of heterogeneity also in the origin of lymphocytes (see recent review by Miller, reference 17). Although it now seems clear that thymus-derived lymphocytes are not the immediate precursors of the producers of circulating immunoglobulins, they seem nevertheless to be necessary for initiating the production. It is still unclear whether antigen-sensitive cells are to be looked for among thymus-derived lymphocytes or among those derived from the bone marrow, the bursa of Fabricius, or possibly yet unidentified sources.

The fact that thymocytes alone can initate GVH reactions (18) strongly suggests that at least thymocytes contain antigen-sensitive cells, albeit on their own they may give rise to cellular immunity only.

Perhaps the most likely possibility is that lymphocytes of either of these derivations have their own antigen-sensitive members.

If this is so there would seem to be no compelling reason why the frequency distribution of specificities should be the same in these different classes. Might it not be, for example, that thymus-derived cells responsible for transplantation immunity and delayed hypersensitivity are more multipotential than the precursors of antibody-producing cells?

A related possibility is that the rather low counts of isohemolytic plaques found by Hildemann and Pinkerton (19) reflect the fact that antigen-sensitive precursors of hemolysin-forming cells with specificity to strong transplanta-

tion antigens are much rarer than the thymus-derived precursors reacting to the same antigens.

SUMMARY

Graft-vs.-host (GVH) reactions were performed in chicken embryos by intravenous injection of adult chicken blood dilutions, and the result was scored by weighing the spleens as well as by karyological identification of host and donor metaphases. From the frequency of detectable signs of GVH reaction when low doses of donor cells were injected into hosts containing one foreign allele of the B locus it is concluded that 1–2% must be a minimum estimate for the frequency of antigen-sensitive cells of a given specificity in this system. It is not found possible to reconcile the findings with the strictest form of clonal selection which postulates a single receptor specificity per clone of antigen-sensitive cells.

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