PRIMARY IMMUNE RESPONSE IN GRAFTED CELLS

Dissociation between the Proliferation of Activity and the Proliferation of Cells*

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(Received for publication 18 January 1967)

Although chicken embryos are in many ways ideal for work on the immunological graft versus host (GVH) reaction, no real use has been made until very recently (1, 2) of the analytical tool offered by the fact that dividing donor and host chicken cells may be distinguished by their sex chromosomes.

The latter fact was demonstrated in a rather preliminary communication by Biggs and Payne (3) who examined a total of 170 metaphases in the spleens of nine female embryos which had been injected with blood from a cock at the 14th day of development and were killed 4 days later. Their material was not identified genetically and there was no assessment of the variability from one embryo to another. In inbred strains of mice chromosomal marker analysis of the enlarged spleens in GVH reactions has revealed a marked variation with strain combination of the extent to which the grafted cells proliferate (4).

The present work aimed primarily at defining the optimal conditions for detection of dividing donor cells in the chick embryo during the course of GVH reaction, which information was needed for a parallel study (2). Thus, the following parameters were investigated: recipient age at grafting, time of kill, dose of grafted cells, and the effect of heavy X-irradiation on the embryos prior to grafting.

It came as an unexpected, but interesting finding that 17-day embryos sustained the multiplication of grafted cells markedly better than 13-day embryos although the latter gave more splenomegaly. In spite of this fact, the proliferation of immunological graft cell activity was considerably higher in the younger embryonic hosts as shown by spleen cell transfer to secondary hosts. The discrepancy between proliferation of donor cells, and proliferation of donor cell activity was so marked that it poses the question: is division of immunologically competent cells in fact necessary to obtain a full-blown primary immune response?

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Materials and Methods

Animals.—Outbred eggs (preliminary experiments) were a cross between closed flocks of Rhode Island Red and Light Sussex (purchased from the Appleby Farm Ltd., Ashford, Kent, England). Highly inbred lines of White Leghorns, I and W, were maintained by Dr. D. G. Gilmour at the School of Agriculture, University of Cambridge, from where we obtained birds for production of F_1 hybrid embryos. Fertility was low, hence we were much restricted in supply of this material. Through the courtesy of Thornbers Ltd., Halifax, Yorkshire, England, ample supplies became later available of birds and eggs which were of known genotype with respect to the powerful histocompatibility and blood group locus B (5–8).

Chromosome Preparations.—Colcemid-treated splenic cells were suspended in 0.95% sodium citrate and kept at 37°C for 20 min. After slow spinning they were transferred to ethyl-acetic fixative (ethanol 3, glacial acetic acid 1) for ½ hr and spun again and transferred to methyl-acetic fixative (methanol 3, glacial acetic acid 1). The cells were then cold spread on to clean microscope slides and stained with 2% propionic orcein. Permanent preparations were made. This technique has given uniformly good preparations and the male cells of ZZ chromosome constitution were readily distinguished from female cells which have only one large Z mediocentric chromosome.

Mitotic Index.—The per cent of spleen cells in mitosis was determined from Leishman-stained smear preparations by counting of at least 1000 cells.

Spleen Assays of Donor Cell Activity.—In the main experiments the primary hosts were 13-day or 17-day B2 × B14 heterozygous embryos injected intravenously with 0.2 ml citrated whole blood from an adult homozygous B14 cock (the same donor being used throughout); in other words, a parental → F_1 hybrid combination with respect to the B locus. In order to correlate the karyological findings 4 days later, when the primary hosts were killed, with the donor cell activity present in their spleens, suspensions were made from the spleens of similarly treated embryos and assayed by injection of 10^6 cells into 14-day secondary B2 × B14 hosts. The latter were killed 4 days later, together with simultaneous control groups injected with known amounts of blood (0.1 ml undiluted, and 0.1 ml diluted 1:10) from the original donor, and the spleen weights were sampled.

The degree of spleen enlargement in the secondary hosts is a measure of the donor cell activity in the spleens of the primary hosts. An example will show how, and on which assumptions, this measure was translated into arbitrary units which permitted a quantitative comparison of the donor cell activity recovered from the two groups of hosts within each experiment.

Experiment 48 (Table IV): 10^6 spleen cells of a pool prepared from the 13-day primary hosts gave on transfer a mean log spleen weight of 1.38. Positive controls injected with 1 unit (0.1 ml of undiluted blood) of immunologically competent cells from the original donor gave a mean of 1.64 whereas controls injected with 0.1 unit gave 1.18 (well above negative controls injected with diluent alone). Since the test system is known to show a linear regression of log spleen weight on the log dose of injected cells (2) it is easy to extrapolate (whether by arithmetic or by use of double-logarithmic graph paper) that 10^6 spleen cells had an activity equivalent to 0.27 units. The assumption is now made that 1 mg of spleen contains on the average 10^6 cells (apart from erythrocytes)—which is at least not an overestimate. Hence the 13-day primary host mean spleen weight of 62 mg represents $62 \times 0.27 = 16.8$ units. Analogously, the 17-day host spleen with a mean of 23 mg represents $23 \times 0.086 = 2$ units.

As a matter of preparative technique: spleen cell suspensions were made by pressing the spleens gently through a stainless steel sieve under dropwise addition of a 20% solution of normal chicken serum in isotonic phosphate-buffered saline. The serum was found to be important both in reducing the number of trypan blue–stained cells and in reducing the mortality on intravenous injection of the cells into the test embryos. Cell suspensions prepared in buffer without serum are often highly toxic.

RESULTS

Preliminary Experiments in Outbred Material.—Table I summarises the results obtained from intravenous injection of RIR × LS embryos with 0.1 cc of citrated whole blood from adult (I × W) F_1 donors of opposite sex (usually male). Consequently these were experiments with donors of constant genotype, whereas the antigenic stimulus provided by the hosts was variable and unknown.

In nonirradiated embryos it will be seen that the per cent dividing donor cells

TABLE I

Normal and Irradiated Rhode Island Red × Light Sussex Embryos Injected with 0.1 ml Citrated Whole Blood of Adult I × W Donors of Opposite Sex

Group No.	Age at grafting	Day of kill	Recipient embryos suitable for cytological analysis			Noninjected control embryos	
			No.	Mean % dividing donor cells* ± SE	Spleen wt‡	No.	Spleen wt‡
Nonirradiated embryos							
	days						
1	13 or 14	4	16	5.3 ± 0.7	1.72 ± 0.04 (= 53 mg)	45	1.03 ± 0.02 (=11 mg)
2	13 or 14	7	11	23.8 ± 8.3	2.07 ± 0.06 (=128 ")	41	1.00 ± 0.02 (=10 ")
3	17	4	14	7.0 ± 1.7	1.44 ± 0.03 (= 28 ")	Same	Same
4	17	7	37	47.3 ± 5.6	1.92 ± 0.03 (= 83 ")	150	1.36 ± 0.01 (=23 mg)
Irradiated embryos (750–1000 R)							
1X	13 or 14	4	26	33.1 ± 5.0	1.18 ± 0.05 (= 15 mg)	15	0.57 ± 0.04 (= 4 mg)
2X	13 or 14	7	4	77.0 ± 8.4	1.29 ± 0.07 (= 20 ")	5	0.75 ± 0.09 (= 6 ")
3X	17	4	14	41.7 ± 8.5	1.18 ± 0.07 (= 15 ")	8	1.04 ± 0.06 (=11 ")
4X	17	7	6	91.6 ± 4.5	1.46 ± 0.05 (= 29 ")	7	1.03 ± 0.06 (=11 ")

* 50–100 metaphases examined in each spleen.

‡ Spleen weights are given as the mean log mg weight ± standard error. Antilog to the mean is given in brackets.

(i.e. the per cent of diagnosed metaphases found to be of donor origin) is on the average higher at 7 days than on 4 days after injection and, for the same time of kill, is higher from grafting on the 17th day of development than on the 13th–14th day. There is, in other words, a ranking order from higher to lower in groups 4, 2, 3, and 1. Furthermore, this order is maintained at a higher level of percentages in the irradiated recipients.

A few additional variations in design were explored with the same material. Thus, 200 metaphases were examined at 24 hr after grafting and not a single donor cell mitosis was found. A group of 12 female chicks were injected at the day of hatching with the standard dose of 0.1 cc of male I × W blood and their spleens examined 7 days later. Again, no donor cell mitosis was found among 600 diagnosable metaphases. A dose-response experiment where 0.6×10^6,

3×10^6, and 15×10^6 I × W separated white blood cells were injected into 17-day embryos killed 7 days later gave the respective scores of 4, 12, and 94% of donor cell mitoses.

We concluded at this juncture that the optimal conditions for diagnosing graft cell mitosis in GVH spleens must be close to injection at the 17th day of development and killing the recipients 7 days later (at day 3 after hatching).

The fact that newly hatched chicks showed absence of dividing donor cells, even when examined 7 days later, must be ascribed to spontaneous maturation of the immune response of the hosts suppressing or eliminating the graft cells.

TABLE II

Female I × W Embryos Injected with 0.1 ml Citrated Whole Blood from a Single Adult W Cock

Age at grafting	Day of kill	No. of embryos	Donor metaphases / Total scored	Per cent	Mean spleen weight		Mean mitotic index	*Total No. of donor mitoses in spleen (× 10^{6})
					Grafted	Normal		
days					*mg*	*mg*		
10	4	4	1/160	0.6	14	6	2.9	2.4
13	4	7	4/400	1.0	47	9	2.7	12.7
14	4	2	0/100	0	54	9	1.7	<9.2
17	4	18	101/677	15	28	13	1.6	67.2
Newly hatched	7	3	68/150	45	77	—	2.5	886.3

* Calculated as $\frac{\text{spleen wt} \times 10^6 \times \text{mitotic index} \times \text{per cent donor mitoses}}{100 \times 100}$, thus equaling 1 mg of spleen to 10^6 cells of the white series; possibly a slightly low estimate.

As to be expected, (cf. Table II) donor cells proliferate well enough in the newly hatched chick when grafted in a parental → F_1 hybrid combination.

Experiments in Inbred Strains.—Our first concern was whether the age-dependant host effect on donor cell division noticed in the outbred material would be repeatable in a parental into F_1 hybrid situation. This is the simplest and clearest way to judge if the age effect is due to immunological maturation of the host or to something different.

It has been shown by Isacson (9), by Solomon and Tucker (10), and by Seto and Albright (11) that the GVH spleen enlargement in chicks has a maximum sensitivity when grafting is made to embryos of 12–15 days of incubation. This is in good agreement also with the spleen weight data of Tables I and II. The reason for this fact, they suggested (9, 10), is that older embryos possess sufficient immunological reactivity to counter the GVH attack by an immune reaction of their own which neutralizes in part the grafted cells.

The data of Table II disprove this hypothesis. Firstly, the 13- to 14-day embryos give more spleen enlargement than the 17-day embryos in spite of the fact that F_1 hosts of any age are for genetical reasons precluded from immune reaction against their parental cells. Secondly, the chromosome data show that grafted cells, far from being eliminated in the older embryos actually multiply better than in the 13- to 14-day embryos. These data, then, bring us back to the negative conclusion which has been stressed before (12); i.e., that we are ignorant about the nature of the host cell proliferation which is elicited by the initial GVH attack. It remains an unexplained fact that this cellular host reaction is best expressed in grafting to 12- to 15-day embryos.

The very low percentage ($< 1\%$) of dividing donor cells found at 4 days after grafting to 10- to 14-day embryos suggested that it may be possible in certain conditions to have a primary immunological response without accompanying proliferation of the immunologically competent cells.

Further support to this conclusion was lent by the fact that a control group of five female I × W embryos which were injected at day 13 with adult I × W male blood showed 4 days later two donor mitoses in a total of 266 diagnosable metaphases; i.e., a frequency (0.75%) similar to the one given by adult W blood in the younger embryos. Hence it is questionable if the few graft cell mitoses found in the grossly enlarged spleens after injection of W blood into 10- to 14-day embryos do at all represent an antigenic stimulation of immunologically competent cells.

Experiments in Blood Grouped Material.—The scarcity of viable I × W embryos necessitated the use of B locus–grouped stock in order to analyze, with appropriate numbers of embryos, the extent to which the primary immune response of a GVH reaction may be dissociated from donor cell multiplication. As a desirable corollary we also obtained complete confirmation of the basic observation in five new and, in respect to the B locus, well defined combinations. The latter fact appears from Table III which also establishes a few additional points:

1. Experiment 49 shows that the inferiority of the 13-day embryo as a host for donor cell multiplication has no lasting effect on the proliferative capacity of the graft cells. When examined 5–7 days later instead of 4, the spleens contain plenty of donor mitoses. In fact, the absolute number of dividing donor cells at the 20th day (13 days + 7) is higher than found at the 21st day in any of the 17-day groups (17 days + 4) of the same combination.

2. Experiment 47 shows a similar result for the group killed at day 20 (14 days + 6), whereas the younger group (10 days + 6) shows that 6 days' sojourn in the host does not in itself suffice for the big mitotic burst. The overall data would suggest that something is lacking in the host environment before about the 17th–18th day of development which is necessary for proliferation of antigenically stimulated immunologically competent cells.

TABLE III

Female Embryos Injected with Citrated Whole Blood from an Adult Cock of Different B Locus Genotype

B group combination (ml blood injected)	Exp. No.	Day of kill	No. of embryos	Donor metaphases		Mean mitotic index	Mean spleen weight	Total No. of donor mitoses in spleen × 10^3	No. of embryos	Donor metaphases		Mean mitotic index	Mean spleen weight	Total No. of donor mitoses in spleen × 10^3
				Total scored	%		mg			Total scored	%		mg	
			13-day hosts						*17-day hosts*					
B14 → B2 × B14 (0.2)	44	4	4	2/300 =	0.67	2.9	49	9.5	2	25/150 =	16.7	3.3	27	149
B14 → B2 × B14 (0.2)	45	4	2	1/100	1.0	0.9	48	4.3	2	32/53	60.5	1.3	23	181
B14 → B2 × B14 (0.2)	46	4	5	4/275	1.45	2.8	51	20.7	2	34/100	34.0	3.3	25	281
B14 → B2 × B14 (0.2)	48	4	4	3/200	1.50	1.6	54	13.0	4	85/200	42.5	1.7	21	152
B14 → B2 × B14 (0.2)	49	4	4	6/200	3.00	2.2	60	39.6						
B14 → B2 × B14 (0.2)	"	5	5	12/184	6.52	3.7	82	198						
B14 → B2 × B14 (0.2)	"	7	5	145/250	58.0	2.4	53	738						
B14 → B2 (0.2)	48a	4	4	2/250	0.80	2.9	58	13.4	4	42/175	24.0	3.3	70	554
B2 → B2 × B14 (0.2)	43	4	3	1/175	0.57	1.4	16	1.3	2	17/150	11.3	2.2	12	29.8
B2 × B14 → B2 × B14 (0.2)	53 61	4	6	1/300	0.33	4.5	13	1.9	6	4/205	1.95	3.9	14	10.6
			10-day hosts						*14-day hosts*					
B2 → B2 × B19 (0.1)	47	6	4	5/400	1.25	4.1	27	13.8	4	85/350 =	24.3	3.8	47	434
B19 → B2 × B19 (0.1)	47	6	1	0/11	0	0.7	48	—	1	11/25	44	1.7	192	1436

Total No. of donor cell mitoses calculated as in Table II. Control spleen weights for 24 13-day hosts (± SD): 9 mg ± 2. Control spleen weights for 17 17-day hosts (± SD): 14 mg ± 2.

3. Experiment 48a shows, most suitably in comparison with the simultaneous Experiment 48, that homozygocity for the foreign B allele gives at least as marked a difference between the mitotic donor cell activity in 13- and 17-day hosts as seen in the hybrid recipients. The 13-day groups of the two experiments are superimposable. The 17-day groups show significantly higher values, for both spleen weight and for the total complement of dividing donor cells, in the B2 hosts than in the hybrid hosts. This difference may conveniently be ascribed to a gene dosage effect, but there is no obvious reason a priori why the latter should operate so much better in the older recipients. The observed difference is, however, in complete support of the notion that no matter how strong is the antigenic stimulus to the grafted cells it does not lead to the expected wave of donor cell mitoses before the host has reached a certain stage of maturation with respect to a still unknown property.

Proliferation of Graft Cell Reactivity.—The crucial test of whether or not the karyological findings reflect the immunological engagement and reactivity of the grafted cells rests with transfer of the GVH reaction to new hosts. Chromosomal markers can only help diagnosing the cells in mitosis, and the reacting cells may not be dividing. Splenomegaly in the primary host is an equally poor guide, because the mysterious host cell proliferation makes it impossible to translate the spleen weight into units of reacting donor cells. Short of a satisfactory plating method for counting the reacting donor cells, we decided to use the transfer technique as described under Materials and Methods. Although the splenomegaly produced in the secondary host is of course also in part, or largely, due to host cell multiplication, this fact is irrelevant as long as the reaction is known to be initiated by cells of the original donor, and the magnitude of the response is quantitated by groups of positive controls injected with known amounts of original donor material.

Table IV shows the results of four identically designed and, as far as possible, identically executed experiments in which the initial inoculum consisted of 0.20 ml (=2 arbitrary units) of citrated whole blood from the same ♂ B14 donor used in all experiments of the combination B14 → B2 × B14. It will be seen that although the absolute estimate of units of donor cell reactivity recovered from the primary host spleens varied considerably from one experiment to another, there was consistently more recovered from 13-day hosts than from 17-day hosts. The best estimate is the ratio of 6.5 based on the pooled data from all four experiments. (It is different, as it should be, from the arithmetical mean of the four ratios).

In Experiment 52, additional information became available from a simultaneously performed transfer of the two primary host spleen suspensions into 17-day B2 × B14 hosts. The latter were killed 7 days later to allow for optimum detectability of dividing cells of the initial donor's descent. The individual results are listed in Table V. They show that (male) donor cells harvested from

TABLE IV

B14 → B2 × B14 → B2 × B14

Recovery of Donor Cell Activity from the Spleens of Primary Hosts Estimated by the Splenomegaly Produced by Transfer of 10^6 Cells to Secondary Hosts of the Same Type

Exp. No.	Primary hosts, killed 4 days after injection		14-day secondary hosts, and positive and negative controls killed 4 days after injection. Mean log spleen weight ± SE (No. of eggs)					
	Age at injection (No. of eggs)	Mean spleen weight	10^6 spleen cells primary hosts	1 unit primary donor	0.1 unit primary donor	20% serum in PBS	Units of donor reactivity recovered from primary host spleens	
	days	*mg*						*ratio*
48	13 (15)	62	1.38 ± 0.03 (10)	1.64 ± 0.08 (5)	1.18 ± 0.02 (5)	0.94 ± 0.04 (6)	16.8	
48	17 (19)	23	1.15 ± 0.03 (10)	Same	Same	Same	2.0	*8.4*
50	13 (5)	34	1.35 ± 0.04 (8)	1.78 ± 0.04 (8)	1.32 ± 0.08 (7)	0.92 ± 0.03 (8)	4.0	
50	17 (9)	23	1.26 ± 0.05 (8)	Same	Same	Same	1.7	*2.4*
51	13 (12)	43	1.47 ± 0.05 (10)	1.79 ± 0.04 (7)	1.51 ± 0.04 (8)	0.97 ± 0.04 (5)	3.1	
51	17 (11)	23	1.26 ± 0.03 (9)	Same	Same	Same	0.3	*10.3*
52	13 (10)	46	1.44 ± 0.05 (8)	1.35 ± 0.03 (5)	1.20 ± 0.08 (7)	0.92 ± 0.03 (5)	183.0	
52	17 (8)	40	1.28 ± 0.04 (8)	Same	Same	Same	13.6	*13.5*
Weighted means	13 (42)	49	1.41 (36)	1.67 (25)	1.33 (27)	0.93 (24)	8.4	
	17 (47)	26	1.23 (35)				1.3	*6.5*

(female) primary hosts, and then transferred to (female) 17-day secondary hosts multiplied at least as vigorously when the primary hosts were 13-day as when they were 17-day embryos. Since the secondary host environment was the same in either group, and since the attained percentage of metaphases which were of donor origin was known to be positively correlated with the number of reactive cells injected (cf. the preliminary experiments), the evidence is clearly a further confirmation of the fact that the 13-day primary hosts contained, after 4 days, at least as many reactive donor cells as did the 17-day hosts, although the donor cells failed to divide to a comparable extent in the former.

TABLE V

♂ B14, Adult Blood → ♀ B2 × B14, 13-Day or 17-Day Primary Hosts Killed 4 Days Later, → ♀ B2 × B14, 17-Day Secondary Hosts Killed 7 Days Later

Primary host age	Findings in secondary 17-day host at 7 days after transfer of 10^6 cells				
	Donor metaphases, Total scored		Spleen weight	Mitotic index	Total No. of donor mitoses in spleen × 10^3
days		%	*mg*		
13	40/40	100	39	2.2	858
13	49/50	98	96	2.6	2461
13	50/50	100	40	2.8	1120
13	41/50	82	86	3.2	2257
17	25/50	50	46	1.7	391
17	29/50	58	76	1.3	573
17	37/50	74	37	4.8	1314
17	10/50	20	52	2.5	260
17	40/50	80	51	2.3	938

Spleen weight of seven normal controls: Mean ± SD 23 ± 3.

DISCUSSION

Optimal Conditions for Karyological Chimera Analysis

The present work began with a methodological problem which we resolved to our own satisfaction, and which does not require much discussion. We were engaged in a study of serial transfer of GVH-induced splenomegaly, initiated with male cells and passaged through female embryos (2). We wanted to use the karyological sex for analysis of the putative chimeras; hence wanted to know when it was best to inject and to kill the embryos. The scoring of thousands of metaphases is no inconsiderable labor, and we must admit to not having looked into all permutations of timing. However, it seemed from the work on outbred material that 17-day embryos were more favorable hosts than 13- to 14-day embryos (which finding was clearly confirmed later in defined combinations of B locus difference when 4 days were allowed before killing).

A 7-day interval produced in either age group a considerable further increase in donor metaphases. The possibility that a longer interval still would have given yet a further increase was not properly explored. Irradiation of the host increased the percentage of donor metaphases (Table I), but it never abolished all host cell division. The net effect on spleen size in irradiated hosts was rather trivial; the radiation-induced atrophy of noninjected control spleens was prevented, but not much over-compensated by the donor cell proliferation. In our previous project we therefore settled for injection of nonirradiated 17-day embryos and killed them 7 days later.

The solution of this practical problem raised two new, and much more interesting ones; i.e., what makes for a good host to the grafted cells, and what is the significance of cell proliferation when it occurs in the primary immune response?

The Age Factor in Embryonic Host Environment

As to the former question, the possibility must first be considered that it is in fact out of place. Could it not simply be that the 10- to 13-day embryo is less antigenic; i.e., is deficient in the B locus antigens which are mainly responsible for inducing the immune response of the graft? If so, the demonstrated lower rate of mitotic donor cell division in the younger hosts would be no reflection of their inadequacy as environment for multiplication of immunologically competent cells. This possibility seems effectively countered by the finding that the 13-day hosts are not only sufficiently antigenic to elicit the GVH response in themselves, but, furthermore, in so doing they induce the grafted cells to more antihost activity in a 4-day period than do the 17-day hosts which, on this hypothesis, should be the more highly antigenic.

Alternatively, as we have suggested already, there may be an environmental deficiency in the younger embryos which allows for no more than a rudimentary proliferation of the immunologically competent cells before the 17th-18th day. This hypothesis has the virtue of fitting all the facts, but it is presented *ad hoc* and there is no direct evidence in its favor. The very claim that young animals are less good hosts for grafted immunologically competent cells than are adults, is by no means new and has been lively debated elsewhere (13). If it holds true for newborn rabbits, as Dixon and Weigle found (14), it might be eminently true for embryos, and the more so the younger they are. It comes easy to mind today to think of the thymus and the bursa of Fabricius in this context, but we have made no attempts so far to test their role.

There is, however, a third possibility which requires consideration. Might it not be that the 17-day embryo appears to sustain the donor cell multiplication better than the 13-day embryo merely because it has a bigger spleen to start with, or because its spleen for reasons other than size is more conducive to "homing" of the injected lymphoid cells? In other words, is it the start, rather than the support of lymphoid cell division which is better in the older embryo?

Although a direct karyological check on this hypothesis is hardly practicable because the concentration of dividing donor cells is just too low in the first 24 hr (cf. experiments with outbred material), the hypothesis has a testable prediction. If true, it should be possible to demonstrate more donor cell activity by the transfer method in 17-day than in 13-day spleens when both are tested early, e.g. at 20 hr after injection of the same dose of donor cells. This point was investigated in two independent experiments, one in the B14 → B2 × B14 combination, the other being B19 → B2 × B19.

In both experiments (to be compared with those of Table IV) the standard dose of 2 units (=0.20 ml) was injected and about 1/50th of this activity was recovered per spleen 20 hr later in both 13-day and 17-day primary hosts. If anything, the recovery was slightly (but not significantly) higher from 13-day hosts.

The hypothesis in question is therefore almost certainly untrue and, short of new evidence, we can offer no better explanation than the postulated deficiency of young embryos with respect to an unidentified factor of significance for lymphoid cell proliferation. (Instead, it might conceivably be due to the presence of a mitotic inhibitor).

Cell Proliferation in the Primary Immune Response

Irrespective of the reason for the demonstrated fact that the GVH reaction in 13-day embryos is associated, for the first 4 days, with less donor cell proliferation than in 17-day embryos, it is of considerable interest that the donor cell proliferation is so badly correlated with increased activity of the grafted cell population. In fact, the correlation is decisively negative.

The proliferation of donor cells (karyological marker) and the proliferation of donor cell activity (spleen cell transfer from primary hosts to 14-day secondary hosts of the same genotype) have been most extensively compared in the B14 → B2 × B14 combination (cf. Tables III-V).

In summary, the findings are: (*a*) that about 1/50th (=0.04 units) of the injected donor cell activity can be recovered per spleen from both 13-day and 17-day hosts at 20 hr after injection of a standard dose of 0.2 ml (= 2 units) of B14-citrated whole blood; (*b*) that the recoverable activity per spleen after a further 3 days (day 4 after injection) has risen to an average of 8.4 units in 13-day hosts, and to 1.3 units in 17-day hosts; i.e., a proliferation of activity in a 3-day period of the order of 8.4/0.04 = 200-fold, and 1.3/0.04 = 30-fold respectively; (*c*) that the total number of dividing donor cells per spleen has concurrently increased to about 16 times as high numbers in 17-day hosts as in 13-day hosts. In other words, about 16 times as many mitoses are accompanied by approximately 6 times less immunological activity when grafting is made to 17-day instead of to 13-day embryonic hosts.

On basis of the presented evidence it seems meaningless to conclude that the rise in immunological activity during the primary response is due entirely, or

even predominantly to multiplication of reactive clones, or indeed to cellular proliferation at all, whether clonal in nature or otherwise. Short of some methodological artefact, which we have been unable to detect ourselves, we rather conclude that multiplication of immunologically competent, or of already primed and reacting cells is detracting from the efficiency with which the immune reaction spreads from involving a few to involving many cells. The fact that the intensity of the immune response, whether primary or secondary, is a reflection of the numbers of cells participating rather than of the height of activity per cell is most convincingly brought out with respect to hemolytic antibody synthesis in all the work with the well-known methods of hemolytic plaque assay. It is the numbers of plaques and not their size which rise and wane.

On the other hand, our data would not justify the categorical conclusion that the increase in immunological donor cell reactivity in 13-day host spleens during days 1–4 after grafting is unconnected with their multiplication. They do after all multiply in detectable numbers. Considering the first four experiments of Table III, it is clear that a mere 0.7–1.5% of donor metaphases correspond to an average total of 12,000 donor cells in mitosis per spleen. This number should perhaps be reduced, allowing for mitoses which are not provoked by antigenic stimulation. However, the control data of Experiments 53 and 61, although fewer metaphases were scored, suggest that nonimmunological mitosis cannot account for all. Furthermore, the controls are in fact only controlling the effect of B locus incompatibility. Let us therefore accept the 12,000 donor mitoses as an upper limit for an estimate, in these four experiments, of the immunologically determined mitotic donor cell activity on the 4th day. Considering now Experiment 49 of the same table, there is an abrupt increase in donor mitoses from the 4th to the 5th day, which would correspond to a mean mitotic cycle of 10.5 hr. If we now apply this estimate of the rate of division to the data of the first four experiments (Table III) it may be calculated that the 12,000 mitoses in the 13-day hosts could have arisen from as few as 22 cells, and the 191,000 mitoses of the 17-day hosts from 344 cells. The inoculum of 0.2 ml of blood contained the order of 2,000,000 lymphocytes.

Similarly, in Experiment 47 (Table III) where 0.1 ml B2 blood was injected into 10-day B2 × 19 and the spleen examined 6 days later, the analogous calculation would suggest that all of the estimated 13,800 mitoses could have been derived from a single cell.

In these calculations (which would fit the clonal selection hypothesis in Burnet's original version) the assumptions are made that the mitotic rate is constant (10.5 hr) during the whole period, and that both daughter cells from each division divide themselves in turn. None of these assumptions may, in fact, be true.

Our most compelling reason for disbelieving the validity of the above calculations comes from experiments in progress which show that substantial splenomegaly (in the B14 → B2 × B14 combination) can in fact be produced with

1/1000th or less of the dose used in the presented data. This merely requires killing the 13-day hosts at day 8 instead of day 4. Clearly, if only a small clone of about 20 cells out of about 2×10^6 injected lymphocytes were the progenitors of the 12,000 mitotic donor cells found 4 days later in the enlarged host spleen, one would at the very most expect splenomegaly to result in one out of 50 eggs injected with a dose which should, on the clonal hypothesis, contain only one reactive cell per 50 doses.

An obvious difficulty for the interpretation of the present data is the possibility that the donor cells might divide for reasons other than direct participation in the antihost reaction although the control data of Table III (Experiments 53 and 61) verify that most of the donor cell division must at least indirectly be ascribed to the GVH reaction. However, this is a difficulty which the present experiments share with more conventional immune reactions provoked by antigen administration to the whole animal. There also it is largely unknown to what extent the dividing cells are actively participating in the specific immune reaction.

The one point which speaks in favor of the possibility that mitotic division of the grafted cells do after all play a role for the increase in recoverable activity over the first 4 days, even in 13-day hosts, is the fact that it is possible from the spleen alone to recover about four times as much activity as went into the whole animal. Still, even this fact could be interpreted differently. It might be a matter of synchronizing the reactivity of a lymphocyte population where all, or most, individuals are genetically competent to react, but are not all in the right physiological phase at the time of injection. Independent reasons for believing that the strong histocompatibility antigens can potentially stimulate the majority of the immunologically competent cells have been given earlier (15, 16).

Concluding the discussion of our present data, they do not rule out that the rise of the primary response of grafted cells against their host is, among other factors, mediated by mitotic division of the donor cells. However, the marked negative correlation found, in comparing the reaction in 13-day and 17-day embryos, between proliferation of cells and proliferation of immunological potency, strongly suggests that there must be alternative ways for recruitment of responding cells.

Completely independent support of this view may be seen in recent experiments by Dr. A. Bussard (personal communication). He finds that peritoneal cells from normal mice can be stimulated to hemolytic plaque formation with sheep red cells and guinea pig complement in a pure in vitro system. Furthermore, the plaque numbers after a 3-day period were very similar to those found by examination of spleens from mice immunized in vivo. Unless it is assumed that he too is deceived by some undetected methodological error, his experiments show not only the in vitro course of a full-blown primary response, but also indicate that the increase in plaque counts over the 3-day period studied is due to something different from the proliferation of plaque-forming cells.

This must be so because the highly viscous gum used in his medium would not allow sufficient migration to enable the daughter cells of a divided competent cell to form more than a single plaque.

How then, if at all, may the present data as well as Bussard's findings be reconciled with the fact that Dr. N. Jerne and coworkers (personal communication) find a large proportion of plaque-forming cells plated from in vivo immunized mice to display mitotic figures?

As far as we can see, it is possible so far to entertain the unorthodox view that mitotic division, when occurring in these circumstances, is essentially a result of allergic damage to the antibody-forming cells, albeit they survive the damage —perhaps through dividing. If this is correct, it would seem a reasonable further proposition that the allergic damage is complement requiring. It is interesting in this context that chicken embryos have been reported to lack complement on the 15th but possess it on the 18th day of development (17). This, then, may explain why mitoses are so much more frequent in our older embryos whilst the recovery of immunologically potent cells is higher in the younger ones. Artificially induced complement deficiency might be valuable conditions in which to study the mitotic rate in plaque-forming cells.

Brent and Medawar's analysis of the normal lymphocyte transfer reaction in guinea pigs (18) is forceful evidence of the fact that the early inflammatory episode of this reaction reflects a "recognition" by which grafted lymphocytes engage in an immunological reaction against host antigens without the need of cellular division. The later flare-up, which can be prevented by immunosuppressive agents they ascribe to graft cell proliferation. The latter reaction would correlate in our experiments with the burst of donor cell mitoses found in 13-day hosts beyond the 4th day after grafting.

SUMMARY

The primary immune response elicited by host antigens in a grafted population of immunologically competent cells has been compared in conditions where the same dose of parental cells were grafted simultaneously to F_1 hybrid embryos of 13 or 17 days of age. The enlarged chimeric spleens harvested 4 days later were analyzed for donor cell proliferation by using the sex chromosomes as karyological markers, and for proliferation of immunological activity by means of transfer to secondary hosts of the same genotype.

Whereas the total number of dividing donor cells were on the average 16 times higher in 17-day than in 13-day hosts, the recovery of immunological reactivity showed a 6- to 7-fold difference in the opposite direction.

The experiments cast doubt on the proposition that cellular proliferation is necessary for development of a primary immune response. They suggest that there exists an alternative way in which a primary immune response may unfold from involving a few to involving a much larger number of cells.

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