FURTHER STUDIES OF THE STIMULATION OF DNA SYNTHESIS IN CULTURES OF SPLEEN CELL SUSPENSIONS BY HOMOLOGOUS CELLS IN INBRED STRAINS OF MICE AND RATS*, ‡

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It was previously reported that a rapid, intense, proliferative response was observed when spleen or lymph node cell suspensions from 2 different rabbits were incubated together, Chapman and Dutton (1). These reactions between homologous cell suspensions are analogous to the reactions between cultures of peripheral leukocytes obtained from 2 human donors as described by Bain *et al.* (2) and Hirschhorn *et al.* (3). They occur in the absence of prior sensitization of the donors and in this sense must be considered a primary response. On the other hand, the rapidity and intensity of the response, and the size of the cell populations involved, are characteristic of the secondary response as seen in cells from hyperimmune animals challenged with the immunizing antigen *in vitro*, Chapman and Dutton (1).

The purpose of this study is to extend these observations to mice and rats where the use of inbred strains makes possible an analysis of the role of genetic factors. The modifications of technique and culture conditions that are necessary in these species are described and some findings which lend support to the concept that the response is immunological in nature are reported.

Materials and Methods

The following inbred strains of mice were obtained from Jackson Laboratories, Bar Harber, Maine: C57BL/10 ScSn (new line), DBA/2, BALB/c, C3H, AKR, A/J, and C57NBL/6, and the F1 hybrids B6AF1 and CAF1. Inbred Lewis, BN, and LBNF1 hybrid rats were obtained from Microbiological Associates, Bethesda, Maryland.

The mice were 8 to 12 weeks old when used, weighing 18 to 25 gm. The spleens yielded approximately 10^8 cells although there were marked strain differences. The rats weighed 120 to 180 gm and yielded 3 to 14 $\times$ 10^8 cells per spleen.

Spleen cell suspensions from a single strain were prepared by forcing the spleens through stainless steel sieves as previously described, Dutton and Page (4), and adjusted to 1.5 $\times$

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10^7 cells/ml. They were incubated in a modified Eagle's MEM suspension medium containing 15 per cent homologous (mouse or rat) serum. The medium contained essential and non-essential amino acids, glutamine, pyruvate, streptomycin, and penicillin, Eagle (5). The ingredients were obtained from Microbiological Associates. A total volume of 2 ml of cell suspension, containing 3×10^7 cells, was incubated in 16 × 150 mm disposable glass tissue culture tubes (Bellco Glass Inc., Vineland, New Jersey) at a 5° slope in an atmosphere of 5 per cent CO_2 in O_2. The proliferative response was measured by the incorporation of tritiated thymidine into the cells. Half a milliliter of medium containing 1 μc of tritiated thymidine with a specific activity of 40 mc/mmole was added per culture. In studies where the kinetics of the response were measured, 12 hour uptake periods were used. In other experiments the thymidine was added at 24 hours and the cells harvested at 48 hours. The cells were washed once in ice cold saline, twice in ice cold 5 per cent trichloroacetic acid, and twice in ice cold methanol as previously described, Dutton and Page (4). The washed cells were dissolved in hyamine and the radioactivity determined by liquid scintillation counting.

The data reported represent the means of triplicate determinations.

Spleen cell suspensions from individual animals or pools from several members of an inbred strain were incubated singly or mixed in varying proportions with homologous cell suspensions as indicated. In all cases the total cell number and volume of medium was kept constant at 3×10^7 cells in 2 ml medium. Departures from these conditions led to serious alterations in the rate of thymidine uptake (see below).

RESULTS

Culture Conditions and Technique.—It was found that the culture conditions for mice and rat spleen cell suspensions were somewhat more exacting than those previously established for rabbits.

The effect of cell density on the rate of thymidine uptake per 10^7 cells, measured in the period 24 to 48 hours after the start of incubation, was investigated. It was found that little thymidine was incorporated at low cell densities (0.5×10^7 cells per ml) in both mice and rat spleen cell suspensions. Above this there was a rapid rise in thymidine uptake per 10^7 cells. Culture densities in excess of 1.5×10^7 cells per ml, however, did not result in any further significant increase and 1.5×10^7 per ml was chosen as the standard condition.

Since the immunological function of the cells was being measured it was considered advisable to avoid the use of heterologous serum which might conceivably cause extraneous stimulatory effects. However, mouse serum is tedious to obtain or expensive to purchase and fetal calf serum was tested as an alternative. Marked increases in thymidine incorporation were obtained with fetal calf relative to homologous serum. This increase was further investigated and the following characteristics established: (*a*) isologous (control) and mixed homologous (stimulated) cultures were not enhanced by a constant factor but by a constant increment, (*b*) the active principle supporting thymidine incorporation was not dialyzable in contrast to the active principle in rabbit serum that supported rabbit spleen cell suspensions, Parkhouse and Dutton (6) and (*c*) the time course of thymidine uptake was characteristic of that seen in antigenic stimulation of cells from previously immunized animals or in stimulation by phytohemagglutinin. Similar effects of fetal calf serum were obtained with both rat and rabbit spleen cell suspensions. The nature and significance of this enhancement of the rate of thymidine incorporation needs further investigation but, in the meantime, it was considered inadvisable to employ it in these studies. Homologous serum was, therefore, used throughout.

In the experiments to be reported, it was important to assess how far variations in the preparation of individual cell suspensions affected the subsequent performance of the cells. A pooled cell suspension was made and aliquots were subjected to various potentially traumatic procedures of the sort that might occasionally occur during the preparation of a cell suspen-

sion. It was found that running the cells up and down a pipette 10 extra times, once through each of 10 different pipettes, or transfer of the cell suspension through 10 culture tubes all produced a slight reduction in thymidine incorporation. Leaving the cell suspension an extra 45 minutes at room temperature before placing in the incubator had no effect but pouring the suspension through 4 additional metal sieves was markedly injurious. All these differences, both major and minor, could be minimized by soaking the sieves, pipettes, and glassware in

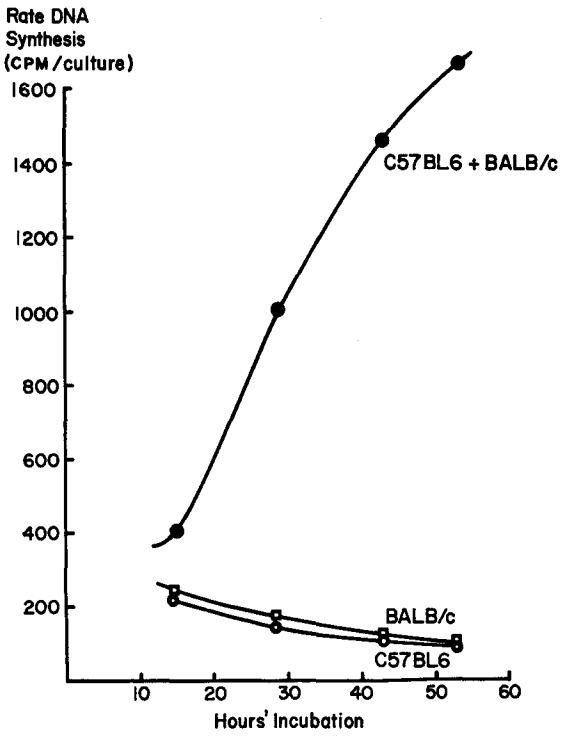

FIG. 1. The time course of the rate of thymidine incorporation in isologous mouse spleen cell suspensions (C57BL/6), ○, and (BALB/c), □, and in mixed homologous cell suspensions (C57BL/6 + BALB/c), ●. The rate of thymidine incorporation was measured by the CPM/culture taken up in 12-hour incubation periods.

phosphate buffered saline for 1 hour at 37°C and then rewashing immediately prior to use. This procedure was followed for the experiments reported here, except in one case indicated below.

Responses in Mixed Cell Suspensions.—

Homologous and isologous combinations: There was a rapid and intense response when cell suspensions of spleens from the different inbred strains were mixed. Fig. 1 shows the time course of the response for a single strain combination in mice; similar results were obtained with rats. The rate of thymidine

incorporation in the control isologous suspension had already fallen to a low level by 14 hours. Clearcut evidence of stimulation could be seen as early as 6 hours on occasion with mice but no attempt has so far been made to measure the early kinetics of the response. Autoradiographs of preparations from the stimulated cultures showed that 1 to 4 per cent of the total cell populations had incorporated thymidine at 48 hours.

In the experiments described above, isologous cell suspensions represented

TABLE I

Responses in Isologous and Homologous Mixes of Mouse Spleen Cell Suspensions

Thymidine uptake in 24 to 48 hour period CPM culture.

	Isologous			Homologous			
	Single	Mean	Mixed	Single		Mean	Mixed
Experiment 1	C57BL/6			C57BL/6,	DBA/2		
	30, 14	22	23	34	342	188	1193
	18, 73	46	28	9	328	169	855
				45	271	158	1258
	DBA/2						
	689, 1624	1156	951				
	618, 419	519	619				
Experiment 2	A/J			C57BL/6,	A/J		
	71, 81	76	89	26	80	53	663
	101, 64	83	49	50	92	71	789
				81	34	68	528
	C67BL/6						
	57, 116	87	93				

pools of cells obtained from the spleens of 3 to 20 mice of the same inbred strain. The progressive decline in the rate of DNA synthesis in such cultures indicated that little or no proliferative response could be occurring in mixtures of cells from the same strain. To confirm this, individual mixes were made with spleens from a single pair of isologous animals and, in addition, individual homologous mixes were made of cell suspensions from a single spleen of one strain with a single spleen of another strain. The results (Table I) indicate that no responses are obtained in individual isologous mixes. It can also be seen that there is relatively little variation between the response of individual pairs of spleens in the homologous mixes.

Responses in different strain combinations: In rats, only one strain combination, Lewis and BN, was examined but a positive response was obtained. Seven strains of mice were tested and responses found in every combination (Table II). The table lists the complete data of the counts obtained in the mixed suspen-

sions and the arithmetic mean of the 2 corresponding separate cultures. This table illustrates the actual variation in thymidine uptake from experiment to experiment. For ease of comparison, the ratios of the mixed cultures to the controls were calculated and the results illustrated in Fig. 2. In addition, the

TABLE II

Responses Obtained in 21 Combinations of Inbred Strains of Mice

Exp. No.	1		2		3		4		5	
Strains*	Mix	Control	Mix	Control	Mix	Control	Mix	Control	Mix	Control
1 + 2	132	36	1298	343	746	202	435	148	1435	246
1 + 3	45	32	380	299	368	123	130	125	283	124
2 + 3	193	47	124	49	305	92	512	108	1990	263
4 + 5	38	18	447	72	469	56	503	72	1901	228
1 + 4	97	18	1493	300	1310	145	693	122	2041	145
1 + 5	219	21	1566	365	1276	195	544	115	2447	190
2 + 4	158	33	53	50	282	64	597	105	1887	284
2 + 5	Not done	Not done	10	115	1253	114	681	98	2265	329
3 + 4	549	18	44	6	330	34	73	81	2795	162
3 + 5	Not done	Not done	1159	70	766	84	611	75	1613	206
1 + 6	112	21	1779	320	1164	175	476	121	2207	291
2 + 6	284	36	2	70	1102	94	651	104	2518	285
3 + 6	301	31	369	26	559	64	763	81	2656	162
1 + 7	93	26	1376	304	938	167	585	122	2077	103
2 + 7	173	41	7	54	137	86	548	105	1826	242
3 + 7	125	37	46	10	288	56	518	86	1405	120
4 + 6	68	18	312	27	808	36	882	78	2800	185
5 + 6	198	20	1344	92	1000	86	630	71	1700	228
4 + 7	53	23	22	11	292	36	602	78	1892	141
5 + 7	26	26	158	76	165	78	119	72	287	186
6 + 7	142	26	17	31	770	58	496	78	1836	142

Experiments 1, 2, and 3 were performed before the introduction of the improved technique for the preparation of the cell suspension (see text).

* Strain; 1 = C57BL 10 ScSn (new), 2 = DBA/2, 3 = BALB/c, 4 = AKR, 5 = C3H 6 = C57BL/6, 7 = A/J.

H-2 loci involved in each mix are listed and the results grouped according to the H-2 locus combination.

Parental-hybrid responses: When mixes are made between cells from 2 inbred strains of mice, the resultant proliferation may be expected to represent the sum of 2 responses, strain 1 against strain 2, and strain 2 against strain 1. F1 hybrid strains were, therefore, used in an attempt to analyze the two components.

Responses were obtained when parental spleen cells were mixed with F1 hybrids (Fig. 3). In further experiments (Fig. 4), spleen cells from 2 parental inbred strains were incubated separately or mixed in various proportions as

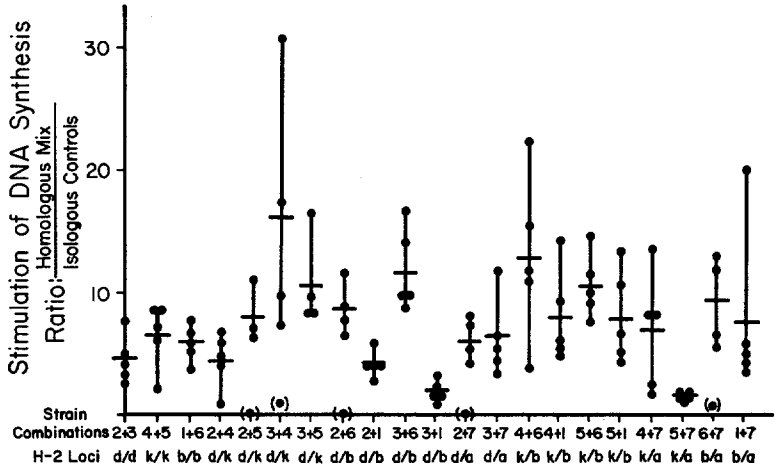

FIG. 2. The figure shows the ratio of thymidine incorporation in homologous mixed cultures to the arithmetic mean of the 2 corresponding isologous controls for 21 strain combinations calculated from the data listed in Table II. The numbers correspond to the strains listed in Table II. The H-2 alleles are listed for each combination and the combinations are grouped according to H-2 alleles. The 5 points for each combination represent the results from 5 separate experiments. The horizontal bar represents the mean of the 5 determinations. The points in parenthesis are considered to represent technical failures and are omitted from the calculation of the mean.

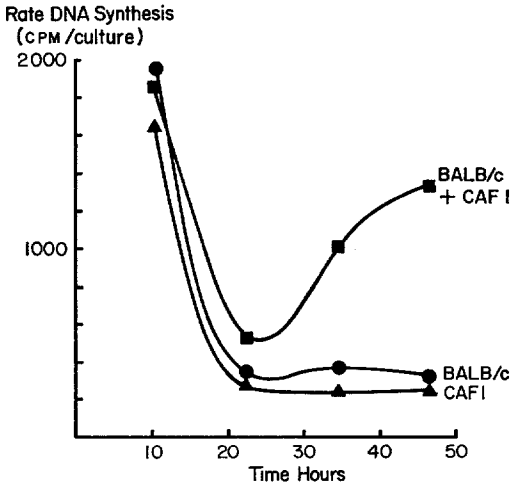

FIG. 3. The time course of the rate of incorporation of thymidine into suspensions of parent (BALB/c), ●; F1 hybrid (CAF1), ▲, or parent + F1 hybrid mixed suspensions, ■. The rate of thymidine incorporation was measured by the CPM/culture taken up in a 12 hour incubation period.

indicated on the abscissa. All cultures contained the same total cell number. The thymidine incorporation measured in the period 24 to 48 hours after mixing is plotted on the ordinate. The thymidine incorporations of similar mixtures of F1 hybrid cells with either parent are plotted on the same graph. It can be seen

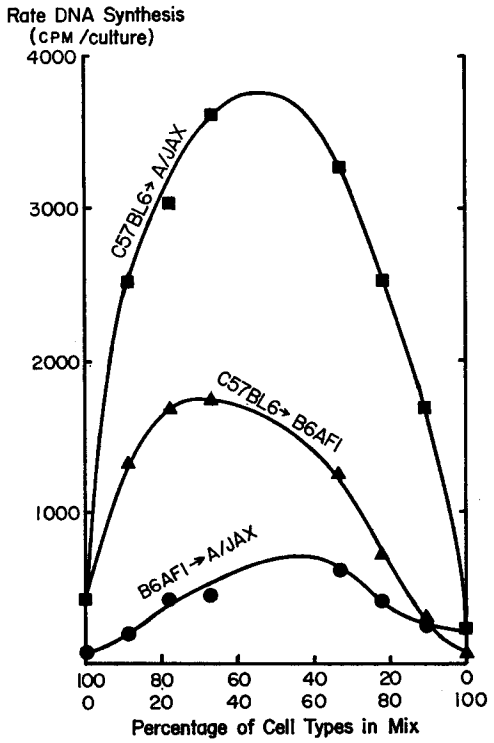

FIG. 4. Analysis of parent *versus* parent and parent *versus* F1 hybrid responses. The thymidine incorporated in the period 24 to 48 hours is plotted against the relative proportions of each cell suspension for the 3 types of mixed suspensions. C57BL/6 diluted out (from left to right) in A/J, ■. C57BL/6 diluted out (left to right) in B6AF1, ▲. B6AF1 diluted out (left to right) in A/J, ●.

that the responses between either parent and the F1 hybrid was less intense than the parent-parent response for the C57BL/6, A/J, B6AF1 system. Similar findings are presented in Fig. 5 *a* for the C57BL/6, A/J, B6AF1 system, as before, and in Fig. 5 *b* for the BALB/c, A/J, CAF1 system. Again, in each system, the parental F1 responses are less intense than the parent-parent response. In addition, Fig. 5 *c*, it is shown that the lowered response is not due to an inherent defect in the functioning of F1 hybrid cells since these give very good responses in mixes with unrelated inbred strains (B6AF1 *versus* BALB/c and CAF1 *versus* C57).

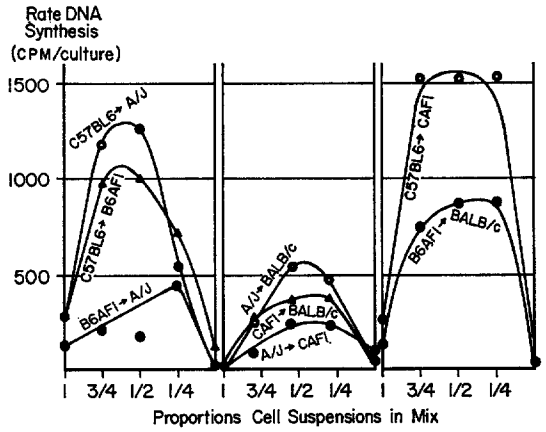

FIG. 5. Analysis of parent *versus* parent and parent *versus* F1 hybrid responses. (*a*) C57BL/6 diluted out (from left to right) in A/J, ○. C57BL/6 diluted out (left to right) in B6AF1, ▲. B6AF1 diluted out (left to right) in A/J, ●. (*b*) A/J diluted out (left to right) in BALB/c, ○; CAF1 diluted out (left to right) in BALB/c, ▲; A/J diluted out (left to right) in CAF1, ●. (*c*) Hybrids responses with unrelated parent. C57BL/6 diluted out (left to right) in CAF1, ○; B6AF1 diluted out (left to right) in BALB/c, ●. Further details, see footnote to Fig. 4.

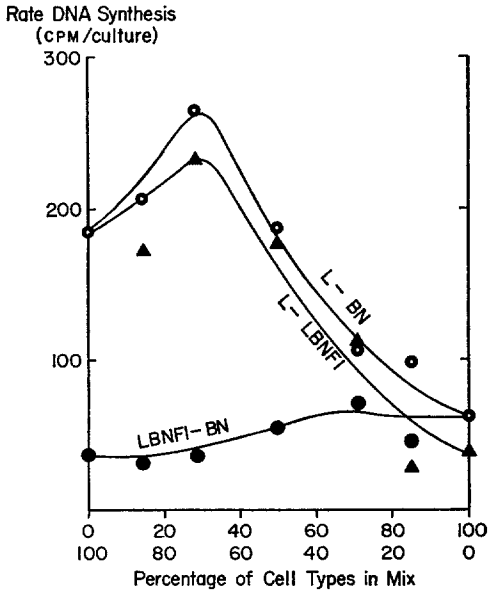

Fig. 6. Analysis of parent *versus* parent and parent F1 hybrid responses in rats. Lewis diluted out (left to right) in BN, ○; Lewis diluted out (left to right) in F1 hybrid, ▲; F1 hybrid diluted out (left to right) in BN, ●. Further details, see footnote to Fig. 4.

Less extensive analyses of the parent-parent, parent-F1 hybrid responses were made in rats. Lewis cells responded strongly against BN (measured by Lewis *versus* F1 hybrid) but BN responded very weakly to Lewis (measured by BN *versus* F1 hybrid), (Fig. 6).

DISCUSSION

The results with mouse and rat spleen cell suspensions establish that the rapid, intense proliferative response seen in cultures of homologous cells is not confined to rabbits and is probably widespread.

As anticipated, no responses were obtained when isologous cell suspensions were mixed.

It might be expected that very uniform responses would be obtained from individual pairs of homologous mixes if the response was dependent solely on genetic differences. The variation seen was not great and could be partially due to unavoidable irregularities in the experimental technique. Occasional failures of the response were noted (*e.g.*, in 5 out of 105 cases in Table II) in otherwise successful experiments. It is possible that these, too, represent technical failures but alternative explanations cannot be ruled out.

All 21 combinations of the 7 inbred strains tested gave positive responses in at least 4 out of 5 experiments. There was, however, considerable variation in the size of individual strain combination responses from experiment to experiment. No clear correlation of the size of the response with strain combination was evident although 2 of the combinations gave consistently low values,—C57BL/10 Sc Sn (new) *versus* BALB/c and C3H *versus* A/J. The first 3 combinations (Fig. 2) did not involve an incompatibility at the H-2 locus. These responses tended to be lower than average but were still sizeable.

The inbred strains used in this study, however, were not suitable for a precise investigation into the possible existence of a relationship between the size of the response and histocompatibility factors. They differed from one another at several known histocompatibility loci in addition to the H-2 and each response can be assumed to be the sum of the response of all the differences of each strain to the other. Instead, Congenic Resistant strain pairs differing at a single locus should be tested against their F1 hybrids. Each locus, however, consists of several alleles, some of which are shared with other loci. Such an analysis could, therefore, only establish a relationship between the response and whole loci and not to the individual histocompatibility antigens controlled by the alleles.

It should be noted too, that if prior environmental factors play a major role in determining the size of the response (as discussed below), patterns of response dependent solely on genetic factors may be obscured.

In the discussion, so far, no comment has been made as to the significance of the response and it is next necessary to ask the question whether it is indeed immunological in nature.

In the studies described, and in the analogous reactions of human peripheral leukocytes, the sole observation has been that of cellular proliferation in cultures containing immunologically competent cells following exposure to genetically dissimilar cells. The response is analogous to the response of hyperimmune cells to antigen in the *in vitro* system and the cellular reactions seen in the whole animal. It does not occur in thymus cell suspensions (1). The discriminatory and adaptive capacities so characteristic of *bona fide* immunological processes have yet to be demonstrated.

The analysis of parent-F1 hybrid interactions, in which it appears that the hybrid does not respond to the parent, lends some support to the concept that the phenomenon is immunological, as will be shown in the following discussion.

The response obtained from mixing cells from 2 inbred strains may be taken to be the sum of 2 responses, each strain against the other. Positive responses were obtained in all cases where parental strains were mixed with their F1 hybrids in both mice and rats. The parental-hybrid responses were always less than the parent-parent response. This was not due to any intrinsic defect of the hybrid cells since these responded well to non-parental strains. The sum of the 2 parent F1 hybrid responses was roughly equivalent to the parent-parent response.

A parent strain will be confronted by the same array of genetically controlled factors in the F1 hybrid that are present in the other parent (providing that these are controlled by dominant genes, as all histocompatibility factors appear to be, see reference 7, page 535), and will respond just as strongly. It is therefore presumed that the reduced response is due to failure of the hybrid to respond to the parent. It should be noted, however, that conclusive proof of this is lacking. In this situation, the hybrid cell does not respond to a different cell (different in that it lacks determinants present in the hybrid) and an element of specificity of the response to different cells is therefore exhibited.

The above argument would be greatly strengthened if it could be shown that cells from 2 mutually tolerant inbred strains did not react to one another but this has not yet been done.

If it is concluded, as suggested above, that this proliferation does indeed represent an immunological response, it is necessary to consider why so many cells take part. Two explanations of its size would appear possible. (*a*) It could be a primary response of unusual intensity involving a significant fraction of the total population of immunologically competent cells. (*b*) It could be a secondary response to tissue antigens which have some component in common with environmental antigens previously encountered by every animal. In the latter case, it would appear necessary to assume that the counterpart of every histocompatibility antigen characteristic of the strains so far tested is present in the environment of every animal. In addition, it would have to be assumed that the

counterparts of other species specific antigens, such as the serum proteins, were absent. This explanation becomes harder to accept with every new strain combination that gives a positive response and it must be confessed that neither explanation is appealing.

The present observations do nothing to resolve the problem but serve to put the alternatives in sharper focus.

SUMMARY

The early proliferative response previously demonstrated in rabbits has now been shown to follow the mixing of spleen cell suspensions from 2 inbred strains of mice or rats. The size of the response is comparable to that seen in cells from hyperimmune animals exposed to antigen *in vitro*. Autoradiographs of cells from stimulated cultures showed 1 to 4 per cent of the total population had incorporated thymidine. Modifications in the conditions necessary for the culture of mice and rat spleen cell suspensions and the measurement of thymidine incorporation are described.

No responses were observed in isologous mixes. The responses obtained on mixing individual pairs of spleens from different strains showed relatively little variation. Responses were obtained in all of the 21 possible combinations between 7 inbred strains of mice. Responses were obtained when parental cells were mixed with their F1 hybrids. Analysis of these responses showed that, in every case, parental-F1 hybrid responses were less intense than those between the 2 parents. It was shown that there was no inherent defect in the ability of the hybrid cells to respond when mixed with an unrelated strain. The results suggested that the hybrid cells made no response to the parent cells although this was not conclusively established. This has been taken as circumstantial evidence that the response is immunological in nature.

The significance of the vigor of the response and the large fraction of the immunologically competent cells that take part is discussed.

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