QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

III. Kinetics of the Response*

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A variety of experimental studies have shown that at the peak of the immune response, the specifically active lymphoid effector cells comprise a surprisingly large number. Thus, murine splenic cells producing hemolytic or hemagglutinating antibody following immunization with sheep erythrocytes amount to 0.1–0.2% of the population (1, 2); and the proportion of lymphocytes in the lymph nodes draining the site of a skin homograft which acquires specific cytocidal activities directed against homologous target cells has been estimated to be 1–2% (3). A similar proportion of adult fowl peripheral blood lymphocytes, when transferred into a homologous environment, are capable of producing pathological lesions—pocks on the chorioallantoic membrane and splenomegaly —which stem from an immunological graft-verus-host reaction (GVHR) (4–7). The rate of appearance of reactive cells in these various immunologic systems has been shown to be essentially exponential; and it has been suggested that most, if not all, effectors arise via proliferative processes following immunological stimulation (see reference 8).

In 1963, Bain and her colleagues (9, 10) described a proliferative interaction which resulted when leucocytes from immunogenetically disparate donors were mixed and placed into culture. Subsequent studies, using inbred strains of rats as cell donors, led to the conclusion that this mixed lymphocyte interaction (MLI) represented an immunologically specific response by competent lymphocytes in culture to homologous cellular transplantation antigens (11–14). Since prior sensitization of the cell donors was not required, there was a strong possibility that the MLI might serve as a useful experimental system for study of the inductive phases of the immune response involving primary contact between antigen and immunologically competent cells.

The simplest, or "minimal," hypothesis of the mechanism of the immune

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response (15, 16) has postulated the existence of "target," antigen-sensitive cells (ASCs),—lymphocytes bearing antigen-specific receptor sites—which undergo proliferation and differentiation to produce populations of effector cells when triggered by specific antigens. The demonstrated specificity of proliferative reactivity in the MLI (12, 13), as well as the possibility that responsive cells in this system might represent ASCs, warranted an extensive study of the behavior of the cells which respond in the MLI, and this forms the subject matter of this report. Special attention has been paid to: (*a*) the latent period, or time before reactivity is initiated, as indicated by cellular proliferation, (*b*) the number of cells which enter mitosis for the first time at the beginning of the proliferative phase as well as at various times thereafter, (*c*) the cell cycle or generation time (*Tc*) of the responding cells, (*d*) whether or not those cells which enter the mitotic cycle divide only once or undergo several sequential divisions, and (*e*) the doubling time (*T*2) of responsive cells—i.e. the time required for the number of proliferating cells at any given time to increase two-fold—and the relationship of this value to the generation time (*Tc*). From these various parameters, estimates were made of the proportion of the initial population of peripheral blood lymphocytes which constitute the responsive, presumably antigen-sensitive, cells.

Materials and Methods

Animals employed as cell donors were rats of the isogenic L, DA, and BN strains and their F_1 hybrids. These strains are known to differ by alleles at the important AgB histocompatibility locus (17, 18).

Cultures were prepared from peripheral blood leucocyte suspensions obtained by dextran sedimentation from whole blood collected by cardiac puncture. Procedures for the preparation and culture of the leucocyte suspensions have been described in detail elsewhere (11). Each culture consisted of a mixture of 2 million lymphocytes—1 million each from a parental strain and an F_1 donor. The use of this experimental design provides a unidirectional response since previous studies have shown that proliferating cells in parental F_1 cultures are almost exclusively of parental origin (11, 12, 14). Control, unmixed cultures, consisted of 2 million lymphocytes from parental or F_1 donors. All cultures were conducted in triplicate unless specified to the contrary, in a volume of 1 ml in 13 × 100 mm disposable flint glass tubes.[1] They were maintained in a humid atmosphere of 5% CO_2-95% air at 37°C. The medium[2] was supplemented with fresh BN serum (10%) prepared on the day of culturing from BN strain donors.

The *proliferative response of the MLI* was assessed by one or more of three procedures:

(*a*) *Incorporation of tritiated thymidine* (*^{3}HTdR*): Following exposure to ^{3}HTdR[3] at specified concentration and duration, cultures were terminated and activity of the DNA fraction assessed by a modification[4] of previous procedures (11) which markedly improved the sensi

[1] RTU tubes No. 7816, Becton Dickinson & Co., Rutherford, N. J.

[2] Eagle's MEM, Schwartz Bio Research Inc., Orangeburg, N. Y. Supplemented with glutamine (2.5 μg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

[3] HTdR: methylthymidine-^{3}H, specific activity 6.7 c/mmole; New England Nuclear Corp., Boston, Mass.

[4] The authors are grateful to Dr. A. W. Kozinski, Department of Medical Genetics, University of Pennsylvania School of Medicine, for this modification.

tivity of the assay. Fluid was removed from the cell pellets by suction, a drop of protein carrier[5] added, and the mixture agitated on a vortex mixer. Proteins and nucleic acids were precipitated by adding chilled 3 M TCA (5 ml) to the culture tubes which were then allowed to stand in the cold for 1 hr. The floccular precipitate was spun down at 1000 *g* at 0°C for 20 min, the supernatant discarded, and the tubes drained vertically. Another drop of carrier was added and the washing procedure was repeated. Following the final centrifugation, the tubes were drained and the pellets dissolved in 2 M NH_4OH (0.5 ml). Aliquots (0.1 ml) of the dissolved samples were transferred to scintillation vials containing glass fiber filter pads, and dried in an oven at 60°C for 2 hr. Scintillation fluid[6] was then added, and the radioactivity measured in a liquid scintillation spectrometer.[7] Generally the range of individual values did not differ from the mean of triplicate cultures by more than 10%. In the few instances where it did exceed 20%, the results were discarded.

(*b*) *Radioautography:* Colchicine-treated cultures were terminated and air-dried slides were prepared according to the standard technique for chromosome preparations of Moorhead et al. (19), using 0.75% sodium citrate as the hypotonic medium. Radioautographs were made by the dipping method, using either Kodak NTB-3 or NTB-2 emulsion. Lower background was obtained with NTB-2. After dipping, the slides were dried horizontally on a cold metal surface which was allowed to come gradually to room temperature during the drying process. This assured uniform emulsion thickness and also reduced "stress factor" background resulting from rapid drying (20). Cracking of the emulsion was minimized by coating the slides with a 1% gelatin solution prior to dipping and by keeping all solutions at the same temperature (15°C) during the developing procedure. After 7 or 14 days exposure in light-tight plastic boxes at 4°C, the slides were processed with Kodak D19 developer, Rapid Fixer, and water rinses. The slides were then stained with buffered Giemsa, and cover slips were mounted with Permount.

(*c*) *Direct enumeration of cells:* The number of surviving, nucleated cells was determined, with a Coulter particle counter (Coulter Electronics, Hialeah, Fla.) at various times after initiating the cultures, by counting nuclear isolates prepared by pronase digestion as described by Stewart and Ingram (21).

Treatment of the Cultures after ^{3}HTdR Pulsing.—Some experiments required that cells be exposed to ^{3}HTdR, washed, and then returned to culture. The procedure adopted in this circumstance was to remove 0.5 ml of medium from each 1 ml culture to be pulsed without disturbing the button of cells at the bottom of the tube. The culture fluids were pooled, filtered, and saved for later use. ^{3}HTdR of specified concentration was added in a volume of 0.1 ml and the cultures gently agitated and returned to the incubator for a specified pulse period. To remove the labeled thymidine, the cultures were diluted with 10 vol of warm MEM[2] containing "cold", nonradioactive thymidine (3 μg/ml), spun down in a centrifuge (500 *g*) at room temperature for 5 min, and the supernatant fluid decanted and discarded. The cells were washed once more in MEM containing cold thymidine and then resuspended in 1 ml of medium consisting of 1 part MEM with 10% freshly prepared rat serum and 1 part of the pooled, filtered, precultured medium.

RESULTS

1. *Magnitude and Kinetics of the Response.*—

In order to obtain detailed information concerning the timing and magnitude of proliferative reactivity in the MLI, mixed cultures of cells from parental and F_1 donors, as well as con-

[5] 2% BSA Cohn fraction V in 10% NaCl. Solubilization of the BSA was facilitated by wetting it with ethanol.

[6] Toluene 3.78 liter; POPOP (1,4-bis[2-(5-phenyloxazol)]benzene) 1.14 g; PPO (2,5-diphenyloxazole) 15.1 g.

[7] Packard Tricarb, Model 3365; Packard Instrument Co., Downer's Grove, Ill.

trol, unmixed cultures, each containing approximately 2 million nucleated cells (90% lymphocytes) were initiated. Groups of cultures were terminated at various times afterwards, and the number of surviving cells in each was determined with a particle counter. Other cultures were exposed to ^{3}HTdR (0.1 μc/ml) for 12-hr periods, at the end of which the level of ^{3}HTdR incorporation was determined by liquid scintillation spectrometry. In the remaining cultures, also exposed to ^{3}HTdR, the number of labeled nuclei and the number of mitotic figures per

TABLE I

Time Course of DNA Synthesis, Mitosis, and Cell Survival in MLI

Group	Culture period of exposure to ^{3}HTdR	^{3}HTdR Incorporation			Labeled nuclei‡ per 1000 cells in BN + BN/DA cultures	Mitotic figures§ per 1000 cells in BN + BN/DA cultures		S/S_0‖ Surviving cells		
		BN + BN/DA	BN	BN/DA		2 hr colch.	12 hr colch.	BN + BN/DA	BN	BN/DA
		*mean cpm**						%	%	%
A	4–20	28	27	35	2	0	0	74.1	73.8	73.9
B	21–32	4	7	6	0	0	0	62.2	61.0	61.9
C	33–44	40	1	2	4		0.7	55.0	53.0	52.1
D	45–56	166	1	4	13		3	48.1	44.3	42.9
E	57–68	471	4	9	46	5.1		42.2	36.9	37.7
F	69–80	873	7	16	120		9.3	38.3	32.0	31.8
G	81–92	1469	40	34	220	16		35.1	25.1	24.9
H	93–104	1521	52	70	191		26	34.3	21.0	20.1

* Mean of 3 cultures; the standard error, SE, of these various means did not exceed 12%.

‡ 1–2 $\times$ 10^3 cells counted/culture; 2–3 cultures examined.

§ 4–10 $\times$ 10^3 cells counted/culture; 2–3 cultures examined; some cultures exposed to colchicine for 2 hr, others for 12 hr.

‖ At T = 0, BN + BN/DA, BN and BN/DA cultures contained 1.98, 1.84, and 1.90 $\times$ 10^6 intact cells, respectively; means determined from 3 cultures; SE of the various means did not exceed 4%.

1000 nuclei, following colchicine arrest for 2 or 12 hr, were determined by radioautographic procedures. These latter indices were assessed by examining 1–2 thousand and 4–10 thousand cells respectively from each culture.

The results of one typical experiment are presented in Table I. During the first culture period studied (group A, 4–20 hr of culture), definite thymidine incorporation had occurred into a few cells. It was clear from this and other experiments that this activity did not persist in the second sampling period (group B, 21–32 hr). At this time, activity in terms of thymidine uptake was 5- to 10-fold less, and mitoses, labeled or unlabeled were never found before the 32nd hr of culture. It was considered that this initial labeling activity did not represent a specific immune response since control cultures behaved in a similar manner, but rather, probably reflected either an adjustment of the blood leucocytes to the culture conditions, or the known low level of DNA synthesis in cells

of normal peripheral blood (22). By the 44th hr after the mixed cell cultures were initiated (group C, 33–44 hr) the levels of ^{3}HTdR incorporation in them were significantly elevated over those in the control cultures, and the first labeled nuclei (0.4%) and mitotic figures appeared. These steadily increased

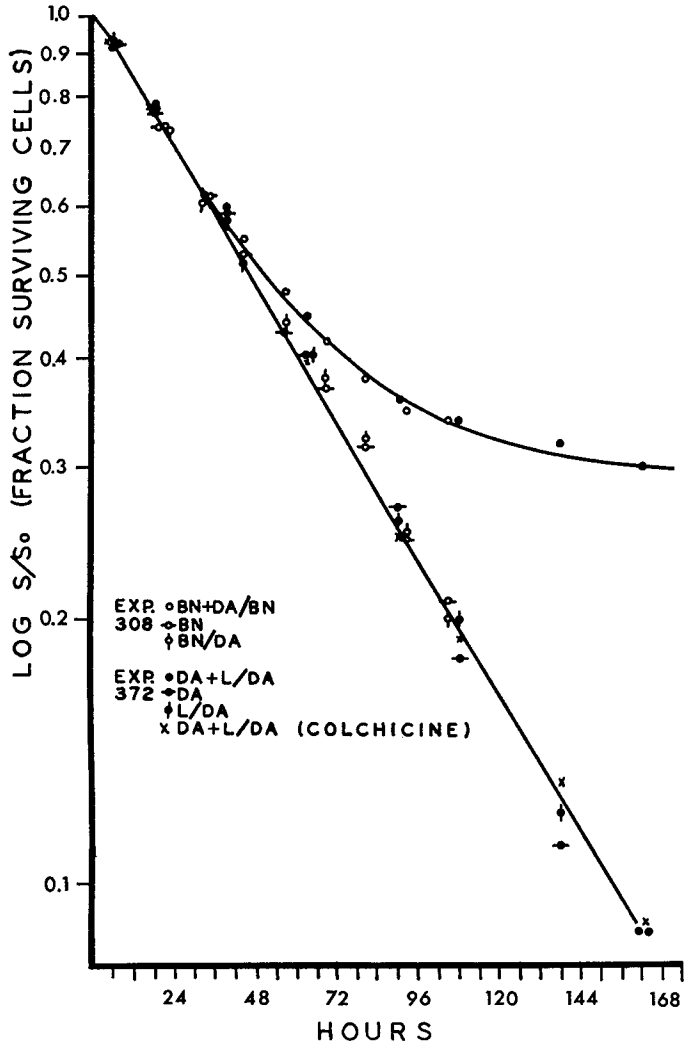

FIG. 1. Proportion of surviving cells at various times in mixed and in control, unmixed cultures. One of the mixed cultures (X——X) of DA + L/DA cells was exposed to colchicine throughout. Each point represents the mean number of surviving cells from triplicate cultures divided by the number of cells cultured at $T = 0$. The standard errors did not exceed 4% of the means.

in numbers throughout the entire culture period (104 hr) of this particular experiment.

The proportion of surviving cells (S/S_0) declined steadily in both mixed and control cultures during the first 48 hr. However, by the 56th hr and thereafter, there were always significantly more cells in mixed cultures, where increased levels of DNA synthesis and mitotic activity were evident, than cells in control cultures of unitary origin. The cell survival data for the experiment presented in Table I and for another similar experiment with another strain combination are plotted together in Fig. 1, and show a significantly increased S/S_0 in mixed cultures following a latent period of approximately 45 hr. However, as is also shown in Fig. 1, the fraction of surviving cells in mixed cultures exposed to

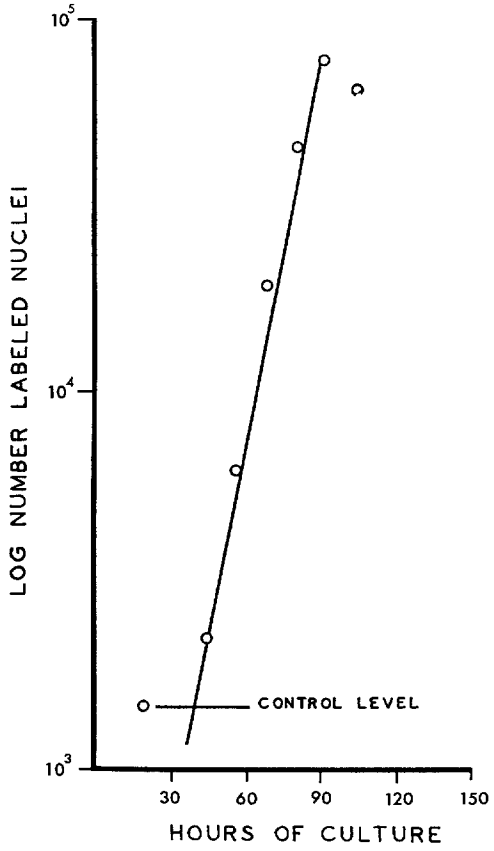

FIG. 2. Relationship of the number of labeled nuclei vs. time of culture. Cells exposed to ^{3}HTdR for 12 hr continuous labeling periods prior to harvesting. Each point was derived from the mean proportion of labeled nuclei in triplicate cultures and corrected for the number of surviving cells.

colchicine, which arrested mitoses throughout the total incubation period, was the same as in control cultures in the absence of colchicine.

Examination of survival curves for both mixed and nonmixed cultures in several experiments revealed a consistent pattern. The half-life (T 1/2) of cells in both types of cultures was approximately 50 hr during the first 2 days, but whereas the surviving cells in the control cultures steadily declined thereafter, S/S_0 of mixed cultures leveled off to a value approximately 30–40% of the number of cells initially committed to culture.

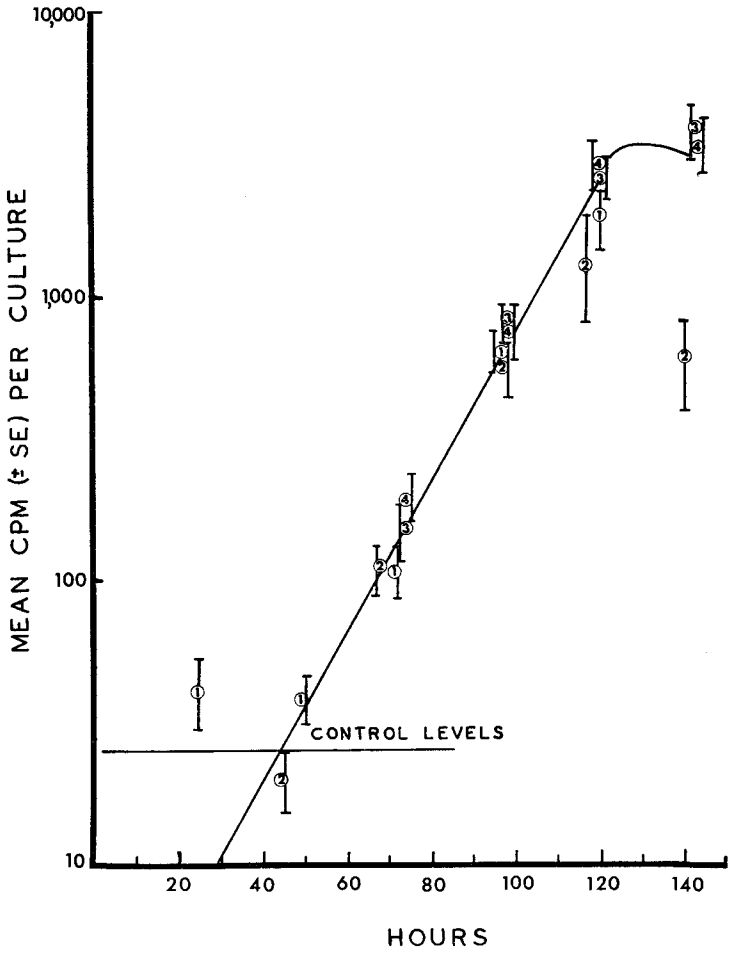

FIG. 3. Relationship of ^{3}HTdR incorporation vs. time of culture. Results from four different experiments (1–4) employing labeling period of 4–6 hr. Each point represents the mean cpm of three parallel cultures. The standard errors did not exceed 10% of the mean.

The number of proliferating cells in mixed cultures at various times was computed from the data in Table I and Fig. 1 by multiplying per cent labeled nuclei by the number of surviving cells per culture. The results, presented in Fig. 2, show an exponential increase over control levels starting at approximately 40 hr, with a doubling time (*T2*) of 9 hr. The proportion of proliferating cells attained during this exponential phase was surprisingly high; at the end of the 4th day of culture approximately 20% of the surviving cells were labeled following 12 hr of continuous exposure to ^{3}HTdR.

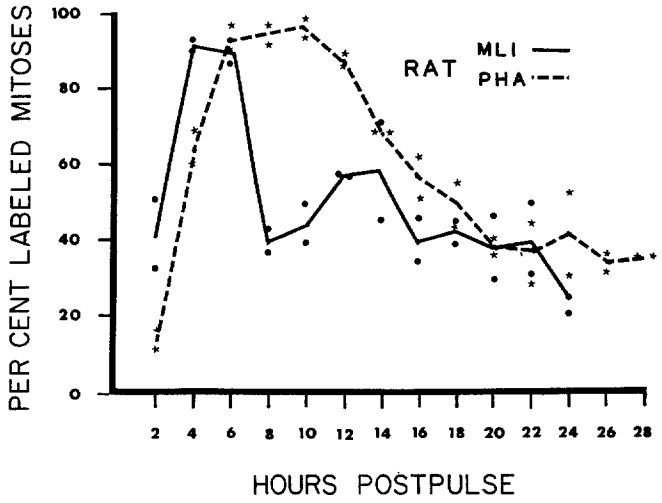

FIG. 4. Cell cycle of typical cultures of BN rat cells stimulated with homologous F_1 cells (●——●) or with PHA (★——★). Individual values for duplicate mixed (●) or PHA-stimulated (★) cultures are indicated; the curve is plotted through their means. *Tc* in *mixed* cultures measured peak to peak, or from comparable points on the first and second mitotic waves is 8 hr; $S = 5$ hr. *Tc* for PHA-stimulated cells exceeds 20 hr and $S = 13$ hr.

Further evidence of the exponential increase of reactive cells with time is shown in Fig. 3, where ^{3}HTdR uptake in counts per minute is plotted against time for four experiments employing labeling periods of 4–6 hr. Following a latent period of 40 hr, the level of ^{3}HTdR increased exponentially with a *T2* of 10 hr. This exponential proliferative phase continued up to 120 hr of culture, at which time proliferative activity began to decrease.

2. *Cell Cycle Time of Proliferating Cells.*—The relationship between the doubling time (*T2*) and the generation time (*Tc*) of immunological effector cells—i.e., that time required for a twofold increase in their number and that time required for them to complete the mitotic cycle—bears directly on the nature of immune response mechanisms. To the extent that *T2* is faster than *Tc*, a "recruitment" of lymphoid cells, not involving linear mitotic descendency from

the initially reacting subpopulation of cells, must occur. It was, therefore, of interest to determine *Tc* for the MLI.

Mixed cell cultures from parental and F_1 strains of rats were initiated and employed for cell cycle analysis on the 4th and 5th day of culture. ^{3}HTdR (0.5 μc/ml) was added for a pulse period of 25 min. Duplicate cultures were terminated at various times (2–28 hr) postpulse after 2 hr exposure to colchicine. Radioautographs were prepared from coded slides (1–2 per culture) and the per cent labeled mitoses determined by blind scoring of 50–100 mitoses per culture. (Rarely, a culture was included in which less than 50 mitoses could be scored.) For

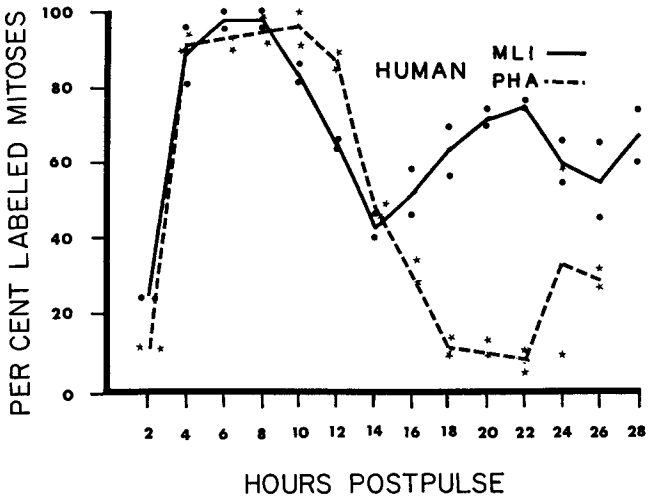

FIG. 5. Cell cycle of human lymphocytes stimulated in MLI (●——●), or with PHA (★——★). Individual values for duplicate mixed (●) or PHA-stimulated (★) cultures are indicated; the curve is plotted through their means. *Tc* = 13–14 hr, *S* = 10 hr in *mixed* cultures, whereas, *Tc* for PHA-stimulated human cells is greater than 20 hr and *S* = 11 hr.

comparative purposes, this cell cycle analysis was carried out on mixed cultures of human leucocytes (23) and on phytohemagglutin (PHA)-stimulated cultures of human and rat peripheral blood leucocytes. The medium for the human cultures was supplemented with serum (10%) freshly prepared from one of the donors.

The results of typical experiments are presented in Figs. 4 and 5, where the per cent labeled mitoses is plotted versus time postlabel, and are summarized in Table II. The data on the rat and human mixed cultures are based on four separate experiments, and the results from PHA-stimulated rat and human cultures are from two and three experiments, respectively. There was no difficulty in identifying the first wave of labeled mitoses in the four different types of cultures, and the length of the DNA synthesis (*S*) period in each instance could, therefore, be ascertained with assurance. In the mixed rat lymphocyte cultures, the *S* period (6 hr) was considerably shorter than that of human mixed cell

cultures (10 hr). In the PHA-stimulated cultures, the *S* period was approximately 11 hr in both instances.

The second wave of labeled mitoses was easily identified in the rat mixed lymphocyte cultures, and thus it was not difficult to estimate the total cell cycle time (Tc = 9 hr), measured either (*a*) from peak to peak or (*b*) from the same point on the ascending curves of the first and second mitotic waves. With

TABLE II

Results of Cell Cycle Analysis

Cultures	Experiment	Donors	Time of culture when label applied	S	*Tc*	
				hr	*hr*	
Rat MLI	199	BN + BN/DA	113	6	9*	9‡
	210	"	110	6	8	9
	347	"	117	7	10	9.5
	366	"	118	5	8.5	8.0
Human MLI	263	DW + JJ	136	10	13.5	13.5
	291	"	137	9.5	16.5	
	345	"	117	9.5	(17)	
	364	DW + PN	142	—	(15)	
Rat PHA	211	BN	64	12	18	
	370	L/BN	96	9.5	(18)	
Human PHA	230	DW	72	11	18	
	216	"	84	10	—	
	369	"	96	11	21	

* Measured at similar points on the ascending curves.

‡ Measured peak to peak.

both the human mixed and the human PHA-stimulated cultures, a second peak was demonstrable, although its limits could not be fixed with confidence. For this reason, Tc estimates for these cultures (14 hr and approximately 20 hr, respectively) were assessed only from comparable points on the ascending curves. Detection of a second wave in PHA-stimulated rat cells was even less reliable. However, it was clear that Tc for these cells exceeded 18 hr.

Comparison of the cell cycle times of PHA and mixed cultures showed that the former were characterized by significantly longer *S* and Tc. Furthermore, G_1 was markedly curtailed or absent in the mixed cultures with a second mitotic wave following closely on the first. Where a second division could be observed in the PHA cultures, on the other hand, a definite G_1 phase was apparent. These

differences in the proliferative behavior of cells cast further doubt on the validity of the use of PHA-stimulated cell systems as in vitro models of immunological reactions.

3. *Fate of the Proliferating Cells.*—Lymphocytes which enter the mitotic cycle may divide once or twice and then become inactive or die; or they may undergo a number of sequential divisions in rapid succession.

To discriminate between these possibilities, a series of mixed cultures of lymphocytes from BN and BN/DA animals was initiated and exposed to ^{3}HTdR (0.1 μc/ml) on the 4th day for a 10 hr period. This labeling period was chosen on the basis of the above studies to exceed the period of one cell cycle, and thus was designed to label all the cells in the cycle at the time of the experiment. After washing, the cultures were returned to the incubator and duplicate cultures were terminated at various times thereafter following 2 hr exposure to colchicine. Radioautographs were prepared, and the per cent labeled mitoses as well as the grain count distribution over the mitotic figures was determined. 100 metaphases per culture were examined.

The results of a representative experiment are presented in Fig. 6, and the findings may be summarized as follows: (*a*) In cultures terminated through the 8th hr postlabel, 100% of the mitotic figures were labeled and, through the 6th hr, these were all heavily labeled, indicating that the exposure period to ^{3}HTdR was sufficiently long to label all the cells in the proliferative cycle. (*b*) The grain count distribution over labeled mitoses at various times up to 56 hr after the labeling period revealed a continuous shift from heavily to lightly labeled cells indicating that once cells entered the proliferative cycle, they proceeded through as many as four or five sequential divisions in rapid succession.

Further studies were conducted to determine whether a significant fraction of cells drop out of the mitotic cycle after one or two divisions instead of continuing to divide. Such "drop outs" would be detectable as heavily labeled interphase nuclei present three or four cell cycle periods after exposure to ^{3}HTdR. Accordingly, mixed cultures, similar to the ones described above, were initiated, pulse labeled with ^{3}HTdR (0.25 μc/ml, 1 hr) on the 4th day, and terminated 12, 24, and 36 hr later, following 12 hr of continuous exposure to colchicine. The grain count distribution over labeled nuclei was recorded (100 labeled cells per culture) and compared to that over labeled mitoses—the latter representing cells known to be still in the mitotic cycle.

The results of this experiment (Fig. 7) show that with increasing time after the labeling period, the general shift in grain count observed over labeled nuclei did not differ from that over labeled mitoses. At 24 and 36 hr postpulse, there were no heavily labeled mitoses or nuclei remaining. This finding also suggests that once cells enter the proliferative cycle in this system they do not drop out after a single mitosis in significant numbers, but rather, proceed through several cycles of cell division.

4. *The Number of Responsive Cells.*—In addition to the progressive decrease in grain count over labeled mitotic figures with time shown in Fig. 6, there was

one other, unexpected, finding. Beginning at 10 hr postlabel—i.e. approximately one cell cycle after the termination of the labeling period—a small, but significant number of unlabeled mitotic figures were noted. These mitoses could not possibly have been derived from heavily labeled nuclei already in the cycle and have a *Tc* of 10 hr since there was insufficient time for label dilution by repeated divisions. Furthermore, it is unlikely that these unlabeled mitoses were derived from cells which were in the cycle prior to or during the labeling phase but possessing an unusually prolonged G_1 or G_2 period. Such cells would have

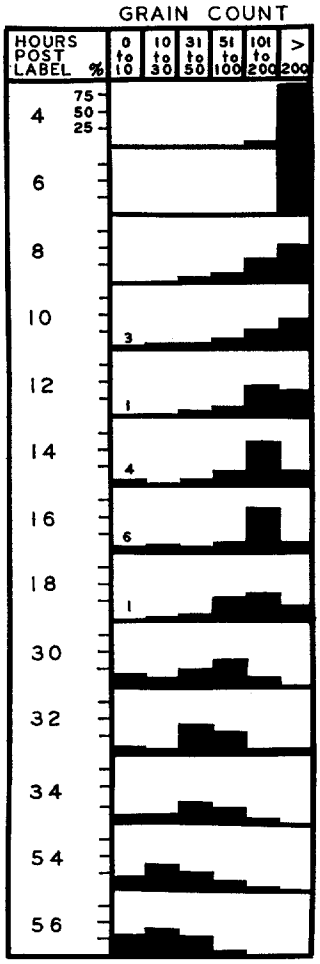

FIG. 6. Frequency distribution of grain counts over labeled mitoses. Cultures were exposed to ^{3}HTdR for 10 hr during the 4th day, then terminated at various times thereafter following 2 hr exposure to colchicine.

not yet entered, or would have completed DNA synthesis (S) before exposure to ^{3}HTdR, and therefore would be equally expected to reveal themselves as unlabeled mitotic figures during the first 8 hr immediately following the labeling period. These were not found at this time. Therefore, unlabeled mitoses appearing more than 8 hr postlabel would appear to be new, hitherto nonproliferating cells which have entered the cycle for the first time, despite the fact that, in this particular experiment this occurred at the beginning of the 5th day of culture—i.e., approximately 80 hr after initiation of the exponential phase (T = 40 hr) of the proliferative response.

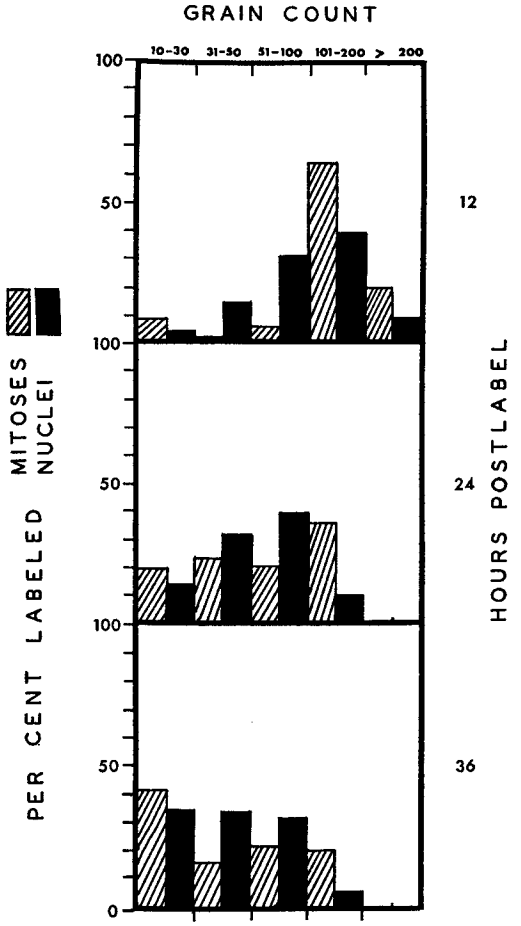

FIG. 7. Comparative frequency distribution of grain counts over labeled nuclei and mitoses. Cultures were exposed to ^{3}HTdR for 1 hr on the 4th culture day and were terminated 12, 24, and 36 hr later following 12 hr of continuous arrest with colchicine.

Notwithstanding the time when they appear, there is the possibility that newly proliferating cells, dividing for the first time, bear some relationship to antigen-sensitive, receptor-bearing target cells (ASCs). Additional studies with mixed cultures of lymphocytes from BN and BN/DA rats were, therefore,

TABLE III

The Nubmer of Newly Responsive Cells/Hour in MLI

Culture period examined	Procedure Employed							
	Unlabeled mitoses			Labeled mitoses		Labeled nuclei		
	Exp. 308	345	346	345	372	372	392	392*
hr								
25–36	0, 0							
37–48	70, 21				70, 85	13, 10, 25		
49–60	150, 130		147					
61–72	210, 50	209		180	254, 257	188, 173, 190	62, 75	26, 20
73–84	120, 160		271					
85–96	225, 84	270		240	103, 204	378, 384, 330 (585, 504, 586)‡		69, 95
97–108	120, 47		233				390 (450)	
109–120	65, 54				95, 96, 191	225, 351, 316	182 (152)	42, 53
121–132	90							
133–144					78, 184, 152	311, 306, 273	165 (75)	32, 34
145–156								
157–168					0, 100, 16	105, 108, 77		
Total responding cells of parental origin, *per cent*	1.3				1.0	3.5	1.0	0.5

* Colchicine from beginning of culture.

‡ Values in parenthesis are from cultures refed on day 3.

designed to confirm and extend this observation and to obtain some estimate of the cumulative number of these new cells which enter the mitotic cycle at various times throughout the culture period. The total number of responsive cells in these cultures represents the fraction of the original 1 million parental lymphocytes which are responsive to the specific homologous antigens presented by the F_1 cells. Identification of cells undergoing their first proliferative cycle in culture was accomplished with the aid of three different radioautographic procedures described below, each designed to exclude proliferating cells *already*

in the mitotic cycle at the time the procedure was initiated:

(*a*) *Unlabeled mitotic figures:* As before, parental and F_1 peripheral blood lymphocytes were cultured and at suitable times throughout the culture period, ^{3}HTdR (0.1 μc/ml) was added to groups of tubes for 12 hr to label all cells then in the proliferating cycle. ^{3}HTdR was then removed by washing and replaced with medium containing colchicine. Cultures were terminated 12 and 15 hr later, radioautographs prepared, and the number of unlabeled mitoses per 2000

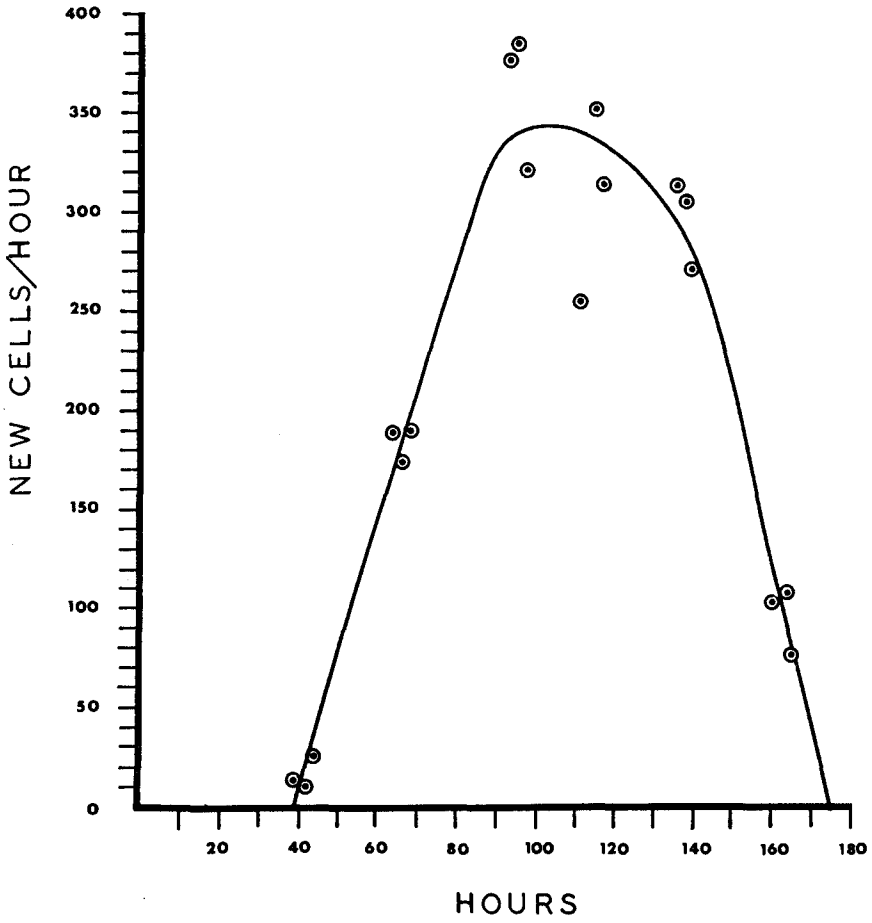

FIG. 8. Rate of new cells per culture on an hourly basis at various times after initiating mixed cultures of BN and BN/DA cells. The new cells were detected and enumerated as labeled nuclei and mitoses (procedure (*c*), see text). Each point was derived from the mean number of labeled nuclei and labeled mitoses from three cultures (2000 cells/culture) exposed to ^{3}HTdR for 2, 4, or 6 hr and then corrected for the fraction of surviving cells at the time they were terminated. The total number of responding cells, based on the area under the curve, amounts to approximately 3.5% of the initial parental cell population committed to culture.

cells determined. The fraction of surviving cells was also determined with the aid of a particle counter. From these data, it was possible to estimate the number of new cells entering the proliferative cycle throughout the culture period, both on an hourly basis and in toto. (See Appendix.)

(*b*) *Labeled mitotic figures:* The procedures outlined above were reversed. Cultures were exposed to colchicine for a period in excess of one cell cycle time to arrest all proliferating cells already in the cycle. ^{3}HTdR was then added (0.25 μc/ml) and allowed to remain in the cultures which were terminated 2, 4, and 6 hr later. In this procedure, no washing was necessary. The new cells, which enter the cycle during the labeling period, are revealed as labeled mitotic figures and their number can be estimated as above. (See Appendix.)

(*c*) *Labeled nuclei:* From the radioautographs of the preceding procedure, the number of labeled nuclei, in addition to labeled mitoses, were determined per 2000 cells. These labeled nuclei and mitoses represented cells in S, G_2, and M of the mitotic cycle for the first time at the time the cultures were terminated and their numbers could be estimated. (See Appendix.)

These three procedures were applied at various times after initiating mixed cultures and the results are summarized in Table III. Each entry in the table represents the results of calculation based on either 3 or 6 individual cultures. Two conclusions are apparent: (*a*) the values obtained for a given period of culture are similar despite the three different procedures employed; and (*b*) the rate of appearance of new cells into the mitotic cycle for the first time varied throughout the culture period. Proliferating cells were first detectable between the 37th and 48th hr, and they entered at an increasing rate through the 4th day of culture, reaching a maximum in the range of 200–400 cells/hr, after which the trend was reversed.

Estimates, based on these data, of the total number of responding parental cells in the original culture inoculum, provided a surprisingly high value. (See Appendix.) The data indicate that 1–3% (i.e. 10–30 thousand/million) of the original parental BN lymphocytes entered mitosis as a result of specific immunologic stimulation by homologous DA strain antigens.

An example of the results of one experiment, based on the scoring of labeled nuclei, is presented graphically in Fig. 8. The position of each point was calculated from the mean number of labeled nuclei, plus mitoses, per 2000 cells each from triplicate cultures.

Further experiments were conducted to determine whether the decreased rate of appearance of newly proliferating cells after 4 days of culture reflected some physiological control mechanism or an artifact due to the exhaustion of nutrient factors from the medium. Half the medium was removed from a number of cultures on the 3rd day and replaced with fresh medium containing 10% serum. The number of new cells/hour was then estimated as before. Although some variation was obtained, in general the preliminary experiments showed that refed cultures were not significantly more active, in terms of the numbers of new cells appearing per hour, than comparable cultures which were not refed (Table III).

These results tentatively suggest that the decreased influx of new cells late in the culture period represents an inherent limitation within the cell population itself.

Finally, to determine whether a continuously dividing population was essential to maintain the entrance of new cells into the system, and to further confirm that these were, in fact, first division cells, another group of cultures were exposed to colchicine from the beginning of the culture period and the number of new cells appearing as labeled nuclei at various times determined. The results of this experiment revealed a slower rate of appearance of first division cells than with the other procedures, but new cells did appear in significant numbers. The total over the culture period was estimated to be approximately 0.5%. (See Table III.) The results of this particular experiment strongly support the interpretation that the new cells described above are not derived from proliferating cells with an aberrant cell cycle.

DISCUSSION

Concerning the kinetics of the immune response as it develops in the intact animal, Makinodan and Albright (8) have described four distinct phases—periods of latency, exponential increase, plateau, and decline—which essentially characterize the amount and rate of increase or decrease of antibody in the blood stream at various times after immunization. Undoubtedly these reflect causative cellular events, although they have been difficult to decipher in studies involving the immune response in the intact animal.

The mixed lymphocyte interaction (MLI) represents an immunological response conducted outside the body of the animal, in a tissue culture system, with lymphocytes from presumably nonimmunized, immunologically normal animals. It possesses the essential characteristics of adaptibility and specificity required of an immune response, despite the fact that the endpoint measured has been cellular proliferation rather than antibody production (12–14). The sequence of phases and the kinetics of its development are not dissimilar to those described by Makinodan and Albright for in vivo systems (8). Furthermore, one study of the immunogenetic circumstances under which both graft-versus-host reactions in vivo and the MLI in vitro occur has found them comparable (14). In both cases, reactivity required immunologic competence of the cell donors and exposure to antigens of a major histocompatibility locus.

It seems obvious, therefore, that the MLI might provide a useful system for examining the initial phases of an immune response. There is, for instance, the possibility of a relationship between the hypothetical antigen-sensitive cells (ASC)—those clones of target cells triggered by antigen to proliferate and then to differentiate into immunological effector cells or to influence their development from a different cell lineage in vivo (8, 15, 16)—and the cells which are stimulated to divide in mixed lymphocyte cultures.

The present studies on the kinetics of the proliferative behavior of cells responsive to a particular histocompatibility antigen system in rats show that there is a latent period of approximately 40 hr after the first exposure of cells to antigen. This is followed by a period of proliferative activity detectable by scintillation spectrometry and radioautography involving a hitherto nondividing cell population. Cells enter their first

S period and appear shortly thereafter as labeled mitoses; then activity in the cultures increases exponentially for many hours with a doubling time (*T2*) of 9–10 hr. During this logarithmic phase, apparently few, if any, of the cells drop out of the mitotic cycle. Rather, they undergo a series of divisions in rapid sequence with a cell cycle time (*Tc*) of 8 hr. This rate of cell division is sufficiently rapid to account for the *T2* of detectably reactive cells solely on the basis of proliferative events, without requiring the involvement of nonlinear recruitment processes.

However, in addition to the cells which first enter the proliferative cycle shortly after the 40th hr of culture, and then proceed through several sequential divisions, new, hitherto nondividing cells continue to enter throughout the culture period, at least up to 160 hr. The cumulative number of these newly responding cells amounts to 1–3% of the original parental lymphocyte inoculum. There is an obvious temptation to equate these newly proliferating cells with the ASCs described above. Certainly, they appear to fulfill the requirements of ASCs as competent cells that are triggered into immunologically specific proliferation. However, it remains to be established whether proliferation in the MLI ultimately results in the production of effector cells.

There is one aspect of equating the responsive cells in the MLI with ASCs which is difficult to reconcile with the clonal selection theory as originally stated by Burnet (24)—their apparently large number. Against antigens such as heterologous red blood cells, the frequency of ASCs has been reported to be of the order of 10^{-5} (25–27), at least three orders of magnitude less frequent than the apparent numbers of newly responsive cells in the MLI. It must be remembered, however, that unlike the cellular reaction to histocompatibility antigens, immunities to heterologous erythrocytes predominately involves the serum antibody response.

The values reported here for reactive cells in the MLI are, in fact, comparable to the fraction of cells capable of producing specific GVH reactions in vivo (4–7). Simonsen has shown that a GVH reaction, detectable by marked splenomegaly, results when fowl embryos are inoculated with as few as 30–50 lymphocytes from unimmunized homologous adult donors. He concluded that a minimum of 1–2% of the peripheral blood population in adult chickens can respond to a given strong histocompatibility antigen (5). Furthermore, Simmons and Fowler have recently shown that the placement of similar numbers of competent lymphocytes on the chorioallantoic membrane results in a destructive focus (4). Making use of the strong histocompatibility H-2 system of mice, Simonsen has shown that deliberate sensitization of cell donors against prospective host antigens does not invariably increase GVH immunologic reactivity of subsequently transferred cells. This finding has been interpreted to indicate that the size of the clone of reactive cells in a normal donor against a particular histocompatibility antigen system is already so large that deliberate immunization does not significantly increase its size (5).

The techniques employed in the present studies to detect newly dividing cells apparently preclude their derivation from proliferating cells already in the cycle and further, they permit an estimate, although crude, of the total number of these responsive cells. The accuracy of three independently measured variables is involved in this estimate: (*a*) the proportion of reactive cells (e.g., labeled nuclei) in radioautographs, (*b*) the fraction of surviving cells, and (*c*) a labeling factor derived from the length of

the *S* period and certain assumptions concerning the behavior of cells passing through the proliferative cycle. Error functions were obtained from the first 2 of these variables, and showed that they would have to be inaccurate by more than 2 standard deviations to affect the estimate of the number of reactive cells/hour 2-fold. The length of the *S* period, 6 hr, as well as *Tc*, 8 hr, compares favorably with values presented for antigen-stimulated lymphocytes cultured in vivo in diffusion chambers (6, 8, and 9 hr respectively) (28). Hence, to double or halve the figure chosen for the labeling factor, as an approximate error limit, would again only change the final estimate of reactive cells 2-fold.

Accepting then, that these newly responsive cells proliferating in the MLI represent an immunologically specific event and that they are in fact ASCs, their surprisingly high numbers might be explained in several ways:

(*a*) Continued proliferation in the MLI represents the absence of physiological feedback control mechanisms so that an abnormal "overshoot" occurs. This is not a likely explanation, since it is the estimate of *new* cells coming into mitosis for the first time under seemingly specific conditions that is disturbingly high.

(*b*) Proliferation in the MLI results from the triggering of a large fraction of different ASCs, each present in a frequency of approximately 10^{-5}, by a large number of different antigenic specificities associated with the AgB histocompatibility locus. This also seems an unlikely explanation since it would require that DA and BN rats, for instance, differ by as many as 100–1000 antigens, all determined solely by the AgB locus, since cells from donors compatible at this locus undergo no proliferation. It should be noted, however, that these strong histocompatibility loci do constitute a multiple alleleic system, and the possible upper limit of antigenic specificities has not yet been determined, (see reference 29).

There is further evidence against this hypothesis. It might be expected that a third strain would share some of the antigenic differences of the first two, and, therefore, BN animals tolerant of DA, for example, should respond to a lesser degree to Lewis cells than normal BN animals—which has not proven to be the case (30). One means of testing this hypothesis would be to determine the fraction of reactive cells that could be stimulated by a simple antigen having a single or few determinants.

(*c*) Proliferation in the MLI is due to "nonspecific" antigen stimulation. Wigzell has raised the possibility that histocompatibility antigens might be highly effective in stimulating ASCs, even those possessing surface receptors with a very low affinity for the antigens. He suggests that because of favorable conditions for cell-to-cell contact in culture, sufficient, threshold, numbers of low affinity receptors may become activated, thereby resulting in the stimulation of the cell (31). Results of studies with cells from specifically tolerant ani-

mals which prove to be specifically unresponsive in culture render this, at best, an unattractive possibility.

(*d*) Reactive cells in the MLI are capable of responding to a number of antigen systems rather than a single one. The studies of Dutton and Mishell (32) with a different immunologic system, however, militate against this view. They showed convincingly that the proliferative response of murine splenic cells to heterologous erythrocytes of two different species involved two different lymphocyte populations. Also, exposure of parental strain lymphocytes in the present system to more than one histocompatibility antigen system results in increased levels of proliferation (30)—which would be expected if different responsive subpopulations were involved.

(*e*) Proliferation in the MLI is specific, but is composed of two or more components—the first being the activation of initially responsive ASCs directly by antigen, and then subsequently these cells exert a "recruiting" influence on nearby cells, bringing them into the mitotic cycle. There is some support for this hypothesis in the observations of McFarland, Heilman, and Moorhead (33). They demonstrated a contact phenomenon, termed *uropodapsis*, between lymphocytes and macrophages and between lymphocytes *inter se*, by which, they postulated, transfer of subcellular material could result in the recruitment and stimulation of other, clonally unrelated cells along a particular immunologic pathway. Such a recruitment phenomenon must of necessity occur only among a population competent to respond. For example, one would not expect to recruit cells from an F_1 donor to react against parental antigen-bearing cells.

One finding, presented above, which makes this a not very appealing hypothesis is that new cells entered the mitotic cycle in only slightly diminished numbers even when the cultures were exposed to colchicine from the outset. It is known that cells die if arrested in metaphase for prolonged periods, and, therefore, it is difficult to imagine the process by which the "recuitment message" is propagated and delivered to cells which respond late in the culture period.

(*f*) Proliferative activity in the MLI stems from a significant amount of nonspecific mitogenesis. As a result of initial specific contact between antigen-bearing and potentially reactive, receptor cells, nonspecific mitogenic agents are released which, in turn, induce the proliferative activity of otherwise unreactive cells. This hypothesis seems very unlikely in view of previous studies which demonstrated with the use of sex chromosome markers that proliferative activity in mixed cultures of parental and F_1 hybrids was totally specific. The responding cells were almost exclusively of parental origin (11, 12).

(*g*) Proliferation in the MLI is initiated and conducted by cells which have had some prior experience with a ubiquitous cross-reacting antigen—i.e., the MLI represents a secondary or recall phenomenon similar to tuberculin-induced proliferation of cells derived from specifically immunized donors (34). Under these circumstances, one might define "strong" histocompatibility antigen

systems—the HLA in man, H-2 in mice, AgB in rats and B in chickens, the first three of which have been demonstrated to be potent mitogens (see references 29, 35, 36)—as those antigens to which a native immunity exists in the intact animal. This might explain the apparently unique characteristic of "strong" histocompatibility antigens. In hosts with preexistent immunities, one might expect to find reactive cells in the frequency of 10^{-2}.

While it is difficult to exclude this possibility, it is an unsatisfactory one. For instance, immunization with strong histocompatibility antigens leads to a prompt and pronounced increase in titers of circulating antibodies from undetectably low levels; and there is every indication that the immunogens which excite the formation of these antibodies are the same determinants operative in transplantation immunity. Mixed cultures conducted with cells from antigen and pathogen-free donors or cultures stimulated with an unnatural antigen might provide some pertinent information.

After examining these possibilities, none of which are fully satisfactory and many of which seem unlikely, to explain what apparently is an inordinate number of primary reacting cells to a given histocompatibility antigen system in rats, we are forced to agree with Simonsen: "that the available number of [reactive] clones should be just about sufficient to cover that little corner of the immunological universe which is made up of a single histocompatibility locus' (5). It seems necessary to conclude that lymphocytes are multipotential, with the capacity to react to several antigen systems although not necessarily at the same time. Although it is difficult to see how such a mechanism of temporary committment could operate, one possibility is that in the absence of antigen, lymphocytes continually alter the specificity of their receptor sites. In the presence of antigens these become fixed and lead to the activation and selection of a large portion of the cell population along a particular immunologic pathway. It is clear that further investigation is required to determine which, if any, of these various possibilities is valid and to determine more precisely the relationship between the proliferative events in the MLI and the initial phases in the mechanism of the immune response.

SUMMARY

The proliferative interaction of cultured rat lymphocytes of immunogenetically disparate origin—the mixed lymphocyte interaction—was employed as an experimental model to examine the initial stages of the immune response mechanism. Using mixed cultures of cells derived from parental strain and F_1 hybrid rats, in which only the parental lymphocytes respond, the following observations were made on the magnitude and kinetics of the reaction.

After initiation of the cultures, there was a latent period of approximately 40 hours during which time no mitotic activity was detected.

This inactive phase was followed by a period of proliferation in which pre-

viously nondividing cells entered the mitotic cycle for the first time. Activity in the cultures, as detected by incorporation of radioactive thymidine and measured by radioautography or scintillation spectrometry, increased exponentially with a doubling time ($T2$) of 9–10 hr.

In this exponential proliferative phase, lasting approximately 100 hr, the dividing cells underwent a series of rapid sequential divisions with a generation time (Tc) of 8 hr, and few, if any, dropped out of the mitotic cycle.

In addition to the cells which first entered mitosis at the beginning of the proliferative phase and then proceeded through multiple divisions, significant numbers of new, previously nondividing cells continued to enter the mitotic cycle during the entire exponential growth phase. The total number of these newly responsive, first division cells throughout the total culture period amounted to 1–3% of the original parental cell inoculum. This is a surprisingly large proportion of peripheral blood lymphocytes with demonstrable reactivity to a particular antigen system, if it is assumed that these first division cells in vitro are functionally related to the hypothetical antigen-sensitive cells which proliferate and differentiate into immunological effector cells. At present there is no entirely satisfactory explanation for this large number of reactive cells in the mixed lymphocyte interaction.

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APPENDIX

Calculations Concerning the Number of Newly Responsive Cells in the MLI.—Because of the possible significance of the number of responsive cells in the MLI which enter the proliferative cycle for the first time, the calculations employed in estimating their numbers are considered sufficiently important to warrant presentation of detailed, worked examples.

Number of new cells per hour: In any given experiment, this value depends on: x, the proportion of newly proliferating cells/2000 nuclei detected by one of three different procedures; S/S_0, the fraction of surviving cells determined with a particle counter; and f, a time factor whose value depends on the procedure employed.

Procedure (a)*: unlabeled mitotic index:* The basis of this procedure is that new cells, dividing for the first time, first appear 10 hr after labeling, and continue to appear thereafter. Using 12 and 15 hr colchicine arrest periods, the proportion of unlabeled mitoses is corrected for the number of surviving cells, then divided by a time factor of two or five to obtain the number of reactive cells/hour.

e.g., with $x = 8/6000$ (3 cultures, 1 slide/culture, 2000 cells/slide) at $T = 108$ hr
$S/S_0 = 0.34$ at $T = 108$ hr of culture
$f = 2$ hr (cultures exposed to ^{3}HTdR for 12 hr, then colchicine for 12 hr before terminating)
Thus, number of new cells/culture/hour = $8/6000\ (1 \times 10^6)\ (0.34)\ (\frac{1}{2}) = 113$ at $T = 108$ hr.

Procedure (b): *labeled mitotic index:* This is the reverse of the above procedure with new cells appearing as labeled mitoses. It is assumed that G_2 lasts 2 hr; therefore, the number of reactive cells is divided by a time factor of two or four for cultures terminated 4 and 6 hr after addition of ^{3}HTdR.

e.g., with $x = 10/6000$ (3 cultures, 1 slide/culture, 2000 cells/slide) $S/S_0 = 0.34$ at $T =$ 108 hr of culture
$f = 4$ hr (cultures exposed to colchicine for 12 hr, then ^{3}HTdR for 6 hr before terminating)
Thus, number of new cells/culture/hour = $10/6000\ (1 \times 10^6)\ (0.34)\ (\frac{1}{4}) = 142$ at $T = 108$ hr.

Procedure (c)*: labeled nuclei:* When ^{3}HTdR is added to a culture of proliferating cells, all

the cells at that moment which are at any point in their S period become labeled. The duration of the S period has been shown to be approximately 6 hr in this culture system. Hence the number of cells per hour entering S, in a steady state, is the number of cells which immediately incorporate ^{3}HTdR when pulsed, divided by the length of the S period. Under conditions where ^{3}HTdR exposure is prolonged, such as for 1–4 hr, significant numbers of cells, which became labeled in S, will appear as labeled nuclei in the postsynthetic G_2 period and will proceed on to accumulate as labeled mitoses in the presence of colchicine. If it is assumed that these labeled nuclei proceed along the various steps of the proliferative cycle at a constant rate, then the number of cells which have entered the cycle on an hourly basis at a particular time equals the number of labeled nuclei, including mitoses, divided by the sum of the S period and the ^{3}HTdR exposure time in hours.

e.g., $x = 50/12{,}000$ (6 cultures, 1 slide/culture, 2000 cells/slide) at $T = 120$ hr

$S/S_0 = 0.31$ at $T = 120$ hr

$f = 8$ hr (6 hr S period plus 2 hr duration of label.) (Cultures were arrested with colchicine for 12 hr, then exposed to ^{3}HTdR for 2 hr before terminating.)

Thus, number of new cells/culture/hr $= 50/12{,}000\ (1 \times 10^6)\ (0.31)\ (\frac{1}{8}) = 162$ at $T = 120$ hr.

The total number of responding cells/culture is derived: (*a*) from the area under the curve of a plot of the number of new cells/hour vs. hours of culture, or (*b*) by multiplying the mean number of new cells/hour by the number of hours that new cells were detectable.