CELLULAR IMMUNITY IN VITRO

Clonal Proliferation of Antigen-Stimulated Lymphocytes*

By WILLIAM H. MARSHALL, ‡, § M.D., FRED T. VALENTINE, ‡ M.D., and H. S. LAWRENCE, M.D.

(From the Infectious Disease and Immunology Division, Department of Medicine, New York University School of Medicine, New York 10016)

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After the observations of Pearmain, Lycette, and Fitzgerald (1) and Schrek (2), that tuberculin could stimulate some lymphocytes from tuberculin sensitive individuals to enlarge and divide in vitro, many similar systems have been studied using bacterial and viral products as stimulants. The common feature of the majority of these reactions is their specificity. Lymphocytes from individuals who have previously been sensitized with the antigen, as evidenced for example by cutaneous delayed hypersensitivity, respond in vitro. On the other hand, lymphocytes from nonsensitive individuals, fail to respond. Because of this correlation, the response in vitro has been considered a secondary immune response (3).

It is assumed that the components of the culture which react specifically with antigen are those cells that later grow into large basophilic dividing cells; these we will term lymphoblasts. This assumption is based on the fact that no other specific cellular events have been observed in lymphocyte cultures, and that serum from sensitive individuals does not confer reactivity on cells from nonsensitive individuals. If this is correct, then immunological memory can be said to reside in such cells and they are thus worthy of careful study.

It should, in principle, be possible to count the number of cells, within a population, which respond to antigen in this way. The question could then be asked whether that number is small enough to be consistent with a "one cell–one immune response" concept such as that developed by Burnet (4) for the clonal selection theory.

The first problem in attempting to determine the number of responsive cells is to discover the mechanism by which the number of lymphoblasts present in a culture

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§ Present address: The Faculty of Medicine, Memorial University of Newfoundland, St. Johns, Newfoundland, Canada.

increases to a maximum after 6–7 days of incubation (see Fig. 5). It has sometimes been assumed that the number of large cells present at the peak of the response represents the number originally stimulated by antigen. Thus a massive but uncoordinated morphologic transformation of a sizable proportion (30% or more in some cases) of the starting population has been envisaged. A second idea, based on the observation of cellular aggregates and rosettes in lymphocyte cultures, is that cell-to-cell interaction in these clusters leads to a continuing "recruitment" into the large dividing cell class. A third possibility is that the increase in number of lymphoblasts is due to repeated division of a few cells.

We approached this problem directly using time-lapse cinematography which enabled us to observe events in a lymphocyte culture for many days. The special feature of these experiments was a small culture chamber about 500 μ diameter whose contents could be visualized in a single microscope field and from which none of the lymphocytes could escape (Fig. 1). Usually 8 frames of film were exposed every minute so that the fate of individual cells and their progeny could be traced throughout the culture period by viewing the projected film.

It is the purpose of this report to present data indicating that the large number of lymphoblasts found after 6 days in antigen-stimulated cultures could arise from a small number of cells that undergo repeated cell division to form clones.

Materials and Methods

Lymphocyte Cultures.—Lymphocyte cultures were prepared from venous blood of healthy volunteers. Blood was either taken into a heparinized syringe containing 8 ml of a 2 mg/ml solution of preservative-free heparin (heparin sodium U.S.P., 151 units/mg, Connaught Medical Research Laboratories, Toronto, Canada) per 92 ml blood, or else into a dry syringe and immediately defibrinated with a glass stirring rod. The blood was allowed to sediment for 1–3 hr at 37°C in tubes; in the case of defibrinated blood, 3 ml dextran (6% w/v in saline, clinical grade, Pharmachem, Bethlehem, Pa.), per 20 ml blood was added. The leukocyte rich supernatant was then removed and mixed with Eagle's Minimal Essential Medium (Microbiological Associates, Inc., Bethesda, Md.), supplemented with 1 ml of a 200 mM solution of L-glutamine per 100 ml medium, and penicillin and streptomycin 10,000 units of each per 100 ml medium, to give 20% plasma or serum and either 0.625 million leukocytes, or in some cases 0.625 million lymphocytes per ml.

The cell suspension was placed in screw capped glass tubes (Demuth Glass Works, New York, N. Y.) 13 mm internal diameter with rounded bottoms, 4 ml per tube. Second test strength tuberculin PPD (purified protein derivative), U. S. P. (Merck, Sharp & Dohme, West Point, Pa.) was dissolved in Eagle's medium and was added at the rate of 2–10 μg per tube. Streptokinase-streptodornase (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y.) was diluted to give SK(streptokinase) 40 units/ml and SD(streptodornase) 10 units/ml; 0.1 ml of this solution was added to each tube. Pokeweed mitogen (Grand Island Biological Co., Grand Island, N. Y.) was added at the rate of 0.01 ml per 10 ml of culture. For mixed leukocyte cultures equal volumes of leukocyte suspensions from different individuals prepared as above were mixed together to give 4 ml final volume per tube (5). Cultures were gased with 5% CO_2 in air, tightly capped, and incubated upright at 37°C.

Mixed leukocyte cultures were prepared from the blood of inbred Lewis and inbred Buffalo rats (obtained from Microbiological Associates). The animals were anesthetized with chloro-

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form and bled by cardiac puncture with a syringe after thoracotomy. The blood was defibrinated with a glass stirring rod and then the red cells were removed by layering the blood on top of a methyl cellulose and "Hypaque" mixture (6). After 2 hr sedimentation, the supernatant serum cell suspension was removed and treated as described above for human cells.

Cell Counting.—For daily total cell counts on tube cultures, equal volumes of cell suspension and 3% acetic acid were mixed and the resulting suspensions counted in four hemocytometer chambers. A new, previously undisturbed culture was taken each day for counts. Differential counts were made on wet preparations in order to avoid the well known nonrandom distribution of cells in smears (7). For these counts a sample of the cell suspension was placed in a Schrek slide chamber (8) and was left undisturbed for half an hour to allow the cells to settle on the lower coverslip. They were then examined at 37°C with an inverted phase contrast microscope using × 100 oil immersion objective and a pale blue filter. 500–1000 cells were counted in samples from tuberculin cultures and 200–500 in samples from the control cultures. Cells were divided into two main groups: (*a*) polymorphonuclear cells, monocytes, macrophages and all dead cells, (*b*) Lymphocytes and enlarged lymphocytes. The lymphocyte category was subdivided into typical blood lymphocytes, lymphoblasts, and intermediate forms. Typical blood lymphocytes presented the same or a smaller image to the eye as did neighboring red cells. Lymphoblasts presented to the eye an image judged greater than that of two red cells, and which showed one or more dark nucleoli. Intermediate forms were neither typical blood lymphocytes, nor typical lymphoblasts. They were usually intermediate in size, nearer that of a small lymphocyte, and contained one or more nucleoli.

Mitosis counts were made after 4 hr exposure of the cells to vinblastine sulfate (Eli Lilly & Co., Indianapolis, Ind.) added at the rate of 0.05 μg per culture. Fixed stained "centrifuged spreads" for these counts were made by the method of Doré & Balfour (9) in a Shandon cell spreading centrifuge (Rainin Instrument Co., Boston, Mass.). The advantages of this method are that the area of the cell spread is small and circumscribed, the cell density in the spread is easily controlled, the individual cells are well spread, and the method of centrifugation disperses clumps so that all the cells in a clump can be clearly examined. We counted all the cells in each spread, which ranged from 7,791 to 17,724 cells. However, of the cells in a suspension spread in this way, only some 10% were recovered in the final preparation. The remainder were presumably lost in the filter paper that is used to soak up the suspending fluid. Counts on wet preparations of the same sample gave only rough equivalence when percentages of lymphoblasts were measured in these and the centrifuged spreads. Therefore, the mitosis counts are a guide to the degree of mitotic activity rather than an absolute measurement.

Cinematography.—The method has been described in detail before (10). Briefly, a Mackaness slide chamber was used, which consists of two circular coverslips mounted in a plastic holder so that they enclose a disc-shaped culture chamber with good optical properties (see Fig. 1). A nylon ring 0.25–0.5 mm internal diameter cut from nylon tubing (Portland Plastics, Hythe, Kent, U.K.) was cemented onto the bottom coverslip with the epoxy resin Araldite (100 parts of resin 502 and 10 parts of hardener 951 from Ciba Products, Co., Summit, N. J.) and served as a microchamber for culturing small numbers of cells. A small volume of a lymphocyte suspension previously incubated in a standard culture tube for 1–4 days was then inserted with a fine pipette into a number of microchambers. The rest of the coverslip chamber was filled with medium from the same culture. When the lymphocytes inside the microchambers had sedimented onto the glass floor, they were examined from below with a Nikon inverted phase-contrast microscope (Ehrenreich Photo Optical, Garden City, N. Y.). A microchamber was selected for filming which contained a very few (sometimes only one) lymphoblasts. Unwanted red cells and small lymphocytes were often removed by inverting the slide chamber for a few minutes; these would sediment out of the microchamber, leaving the lym-

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phoblasts still adhering to the glass. The fate of cells in the chosen microchamber was then recorded by 16 mm time-lapse cinemicrography equipment, assembled by Sage Instruments, White Plains, N. Y., and adapted to take 1200 ft of film. Framing rates of 2–9 frames per min were used with ½ sec exposure onto Kodak Plus X reversal film. The films were viewed on an analyzing projector which incorporated single frame viewing and a frame counter. Thus, the time between filmed events could be accurately measured by counting the intervening frames on the film. In these experiments, cell generation time was taken to be the interval between successive observation of telophase in a single clone.

RESULTS

In some 30 experiments, lymphocyte cultures stimulated by various methods were observed by time-lapse cinematography. Since it had been shown that

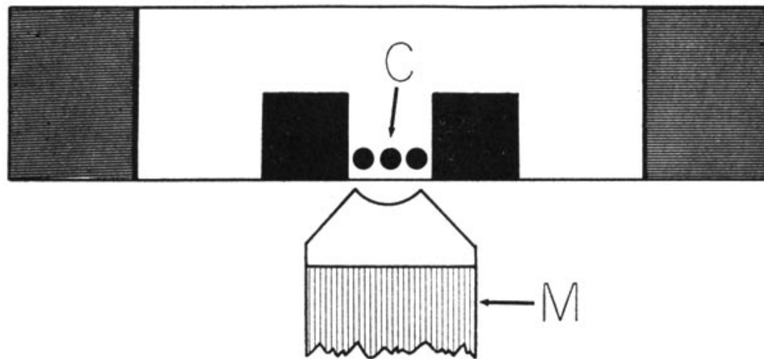

FIG. 1. Diagram of the method of filming cells in a coverslip chamber. The chamber is seen in section with the objective of an inverted microscope (M) focused on the cells growing in the confines of a microchamber. The microchamber is made by cementing a nylon ring onto the lower coverslip.

good stimulation depended on high cell density (11), at least in the first 24 hr (12), the cultures were always started in conventional tubes where the cells would sediment into a small dense button. Although good survival and repeated division of the cells was occasionally found after only 24 hr preliminary incubation, proliferation of lymphoblasts was generally poor if the preliminary period of incubation in tubes was less than 3 days. This experimental design has yielded information about the capabilities of blast cells in terms of generation time and clone size. It must be stressed, however, that this method has not given a total picture of events in a lymphocyte culture from the time of addition of antigen.

Behavior of Cells Filmed in Microchambers.—Lymphoblasts behaved in the same general way as those produced from small lymphocytes with phytohemagglutinin as described previously (10). That is to say, they usually showed a lymphocytic type of motility; sometimes moving about the culture chamber and sometimes remaining anchored in one spot by a "foot appendage" (13). In clumps, a more rounded, yet still motile form was often seen. During the final

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days of a culture period, cells often became rounded up and remained immobile for many hours; however, even in this state they were seen to grow large and divide.

Clumping on a microscopic scale was frequent; clusters of 4–20 cells were commonly observed. Clumping took place because cells in the same clone adhered to one another and because colliding cells tended to adhere to each other after chance contact.

Lymphoblasts tended to stick to glass. The adherence was minimal compared to that of monocytes or macrophages but was greater than that of small lymphocytes. We made use of this property frequently when making preparations for filming. Inversion of the slide chambers would empty the microchamber of red cells, small lymphocytes, and debris, leaving the lymphoblasts stuck to the glass; when the slide was returned to its former position, the lymphoblasts and phagocytic cells were the only remaining occupants of the microchamber.

Lymphoblasts divided to form more lymphoblasts, which in turn grew large before they divided (Figs. 6 and 7). Lymphoblasts were never seen to divide to produce cells as small as typical small lymphocytes. Although the diameter of lymphoblasts did decrease somewhat over several generations, the cells up through the 8th day of culture did not revert to small lymphocytes under the conditions of these experiments.

Generation Time.—Altogether 301 generation times were measured by time-lapse cinemicrography. The majority of these (251) were obtained from the 10 experiments with tuberculin in which lymphocytes from 6 different donors were used. Generation times were measured both by observation of individually traced cells and by calculating mean times between successive mitotic waves in clones where individual cells could not be followed. The data are individually displayed in Fig. 2 and Table I. Generation times varied between 7.5 and 38 hr, with most values ranging between 8 and 13 hr. The pooled data are shown as a histogram in Fig. 3. The generation time varied at least as much within a single clone as between experiments or between individuals. There was a tendency for the generation time to be long in the early phases of clone development, to become progressively shorter down to 8–10 hr, and then to lengthen as the clone reached the 64 cell stage (e.g., see Fig. 2*g*).

The observations made either with SK-SD (Fig. 2*k*) or with pokeweed extract (Fig. 2, *o*) show no clearcut differences from the results obtained with tuberculin. The measurements made in the mixed leukocyte reaction (Table I and Fig. 2*l*, *m*) using three different pair combinations, have an average generation time of 15.0 hr and a range from 8.5 to 22 hr. Finally, 10 generation times in a rat mixed leukocyte culture were between 8.1 and 16.5 hr (Fig. 2*n*) and were comparable to the human measurements.

Clone Size.—Due to clumping, it was not always possible to follow the fate of single cells on the projected film. Thus, continuity was lost and a complete picture of the development of a clone could not be built up. It is for this reason

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that the diagrams (Fig. 2*a–o*) often display small clones. However, it must not be imagined that these small clones did not develop further; most of them are really fragments of much bigger clones. A typical example illustrating these difficulties is shown in Fig. 2*h*; in this microchamber 3 lymphoblasts were

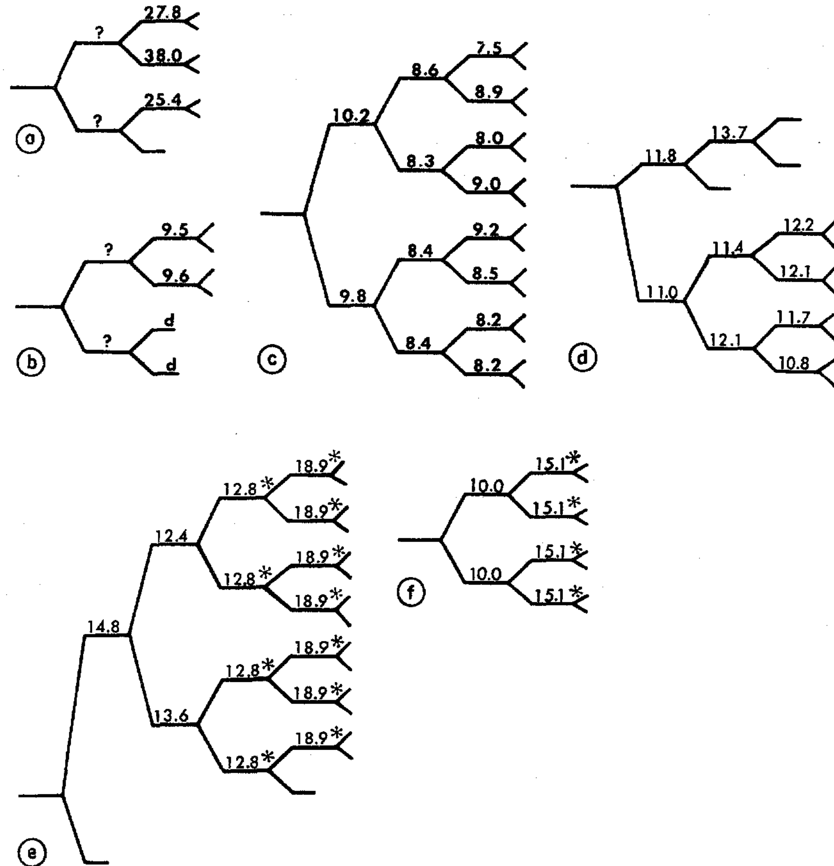

FIG. 2. Diagrams *a–o* are reconstructions of the fate of all lymphoblasts that were followed for more than a single cycle. The horizontal line at the left of each diagram represents the original lymphoblast; following across towards the right, each division of the line respresents a mitotic division of the cell. Generation times are written in hours over the appropriate lines. Those times marked with an asterisk are average times for a group of cells where it was impossible to follow the fate of single cells but where the group itself was discrete and all the mitoses within the group were clearly seen. Dotted vertical lines are drawn when continuity was lost in following the development of the cell line; dividing cells to the right of the line were derived from cells shown on the left but the delineation of a more precise relationship was not possible. *a*. Subject Luc with tuberculin (Exp. 37); *b*. Subject Sim with tuberculin (Exp. 25); *c*. Subject Sim with tuberculin (Exp. 25); *d*. Subject Luc with tuberculin (Exp. 26); *e*. Subject Sim with tuberculin (Exp. 29); *f*. Subject Sim with tuberculin (Exp. 29).

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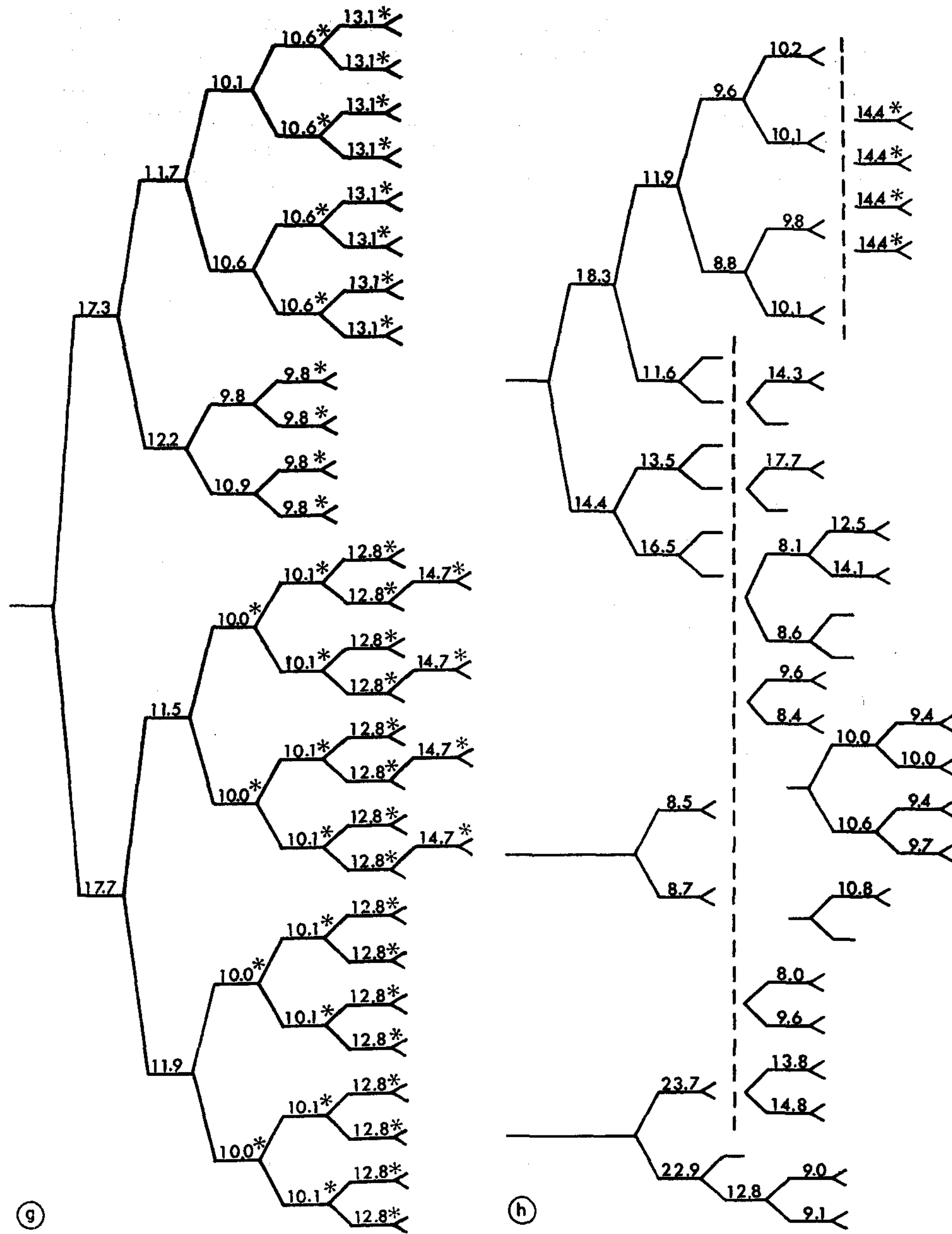

FIG. 2. *g*. Subject Sim with tuberculin (Exp. 29); *h*. Subject Zol with tuberculin (Exp. 30).

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present initially and they divided repeatedly to form 3 large clones, yet only partial reconstruction of events was possible as the cells frequently became confused with each other in clumps. The important part of these observations, therefore, is that where it was possible to trace single cells right through a series of divisions, large clones were observed to develop. When cinematography conditions were good and the microchambers were not overcrowded, it was usual

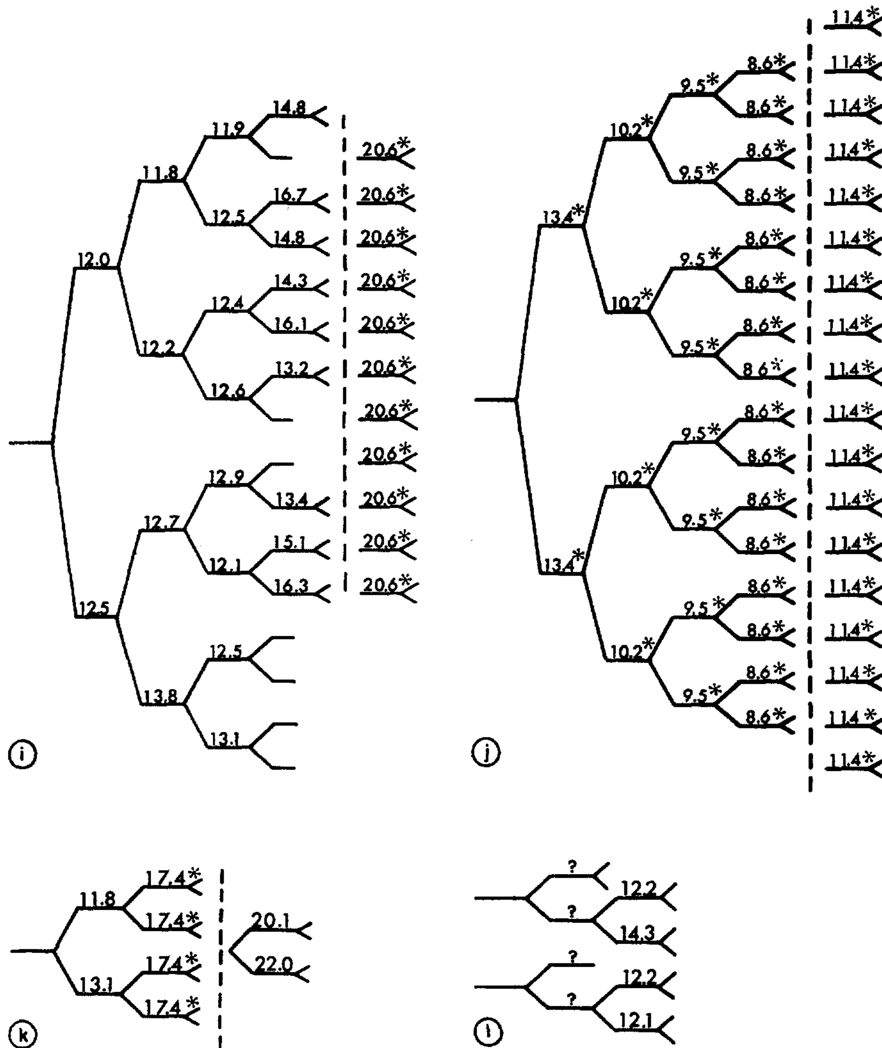

FIG. 2. *i*. Subject Cam with tuberculin (Exp. 35); *j*. Subject Sim with tuberculin (Exp. 13); *k*. Subject Sla with SK-SD (Exp. 32); *l*. Mixed leukocyte reaction (Exp. 18).

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to see clones of 16 cells develop, and often clones of 32 cells were traced. On several occasions, 64 or more cells were seen to develop from a single cell; however, they had been cultured for 6–8 days by this time and many cells were dying.

Correlation with Events in Standard Tube Cultures.—Having demonstrated

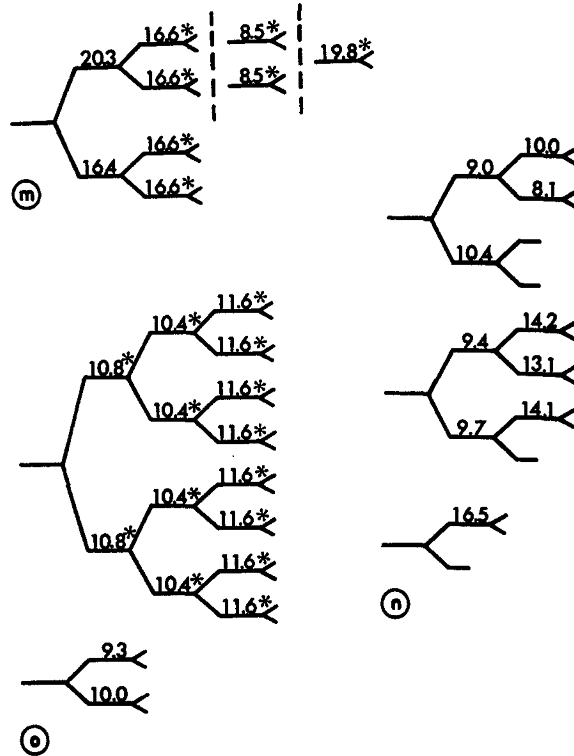

FIG. 2. *m*. Mixed leukocyte reaction (Exp. 41); *n*. Rat mixed leukocyte reaction (Exp. 28); *o*. Subject Leb with pokeweed mitogen (Exp. 27)

that the increase in cells observed in microchambers resulted from clonal proliferation, it was next of interest to relate these measurements, made on single cells, to the proliferative events in a large population of cells grown in standard culture tubes. Before comparing doubling times and increases in total number of lymphoblasts in the two situations, it was necessary to detect the time of onset of mitotic activity in tube cultures. If an increase in the number of lymphoblasts occurred in the absence of mitoses, then clonal proliferation would have to be excluded as the cause of the increase; if, however, cell division began early after the addition of antigen, there would then be justification for considering clonal

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proliferation as a possible mechanism to account for subsequent increases in the number of lymphoblasts.

Mitosis Counts.—Mitoses were counted on centrifuged cell spreads made after

TABLE I

Isolated Measurements of Generation Time in Cells, Followed only for One Cell Cycle

Tuberculin stimulated cultures		
Exp.	Subject	Time*
		hr
8	Ros	9.4
		10.4
8	Ros	14.3
		14.4
13	Sim	8.3
15	San	12.6
19	Luc	9.7
		9.1
"	"	12.7
		13.8
"	"	12.4
		13.7
"	"	11.2
		12.9
19	Luc	10.4
		10.7
"	"	9.9
"	"	11.7
		11.8
"	"	11.9
		12.8
	Mixed leukocyte cultures	
18	M + SL	14.0
		13.5
20	M + J	22.0

* Two entries for time indicate that the two generation times in each case were observed in cells derived from the same cell.

exposing cultures to vinblastine, as described under methods. Counts of mitotic figures were performed daily for 4 days on tuberculin-stimulated and nonstimulated cultures prepared from one individual (Sim) with intense cutaneous reactivity to tuberculin. A different culture was used for each estimation and was discarded after sampling. The results are given in Fig. 4 and in Table II, and show that the beginnings of a mitotic response occurred as early as 48 hr after

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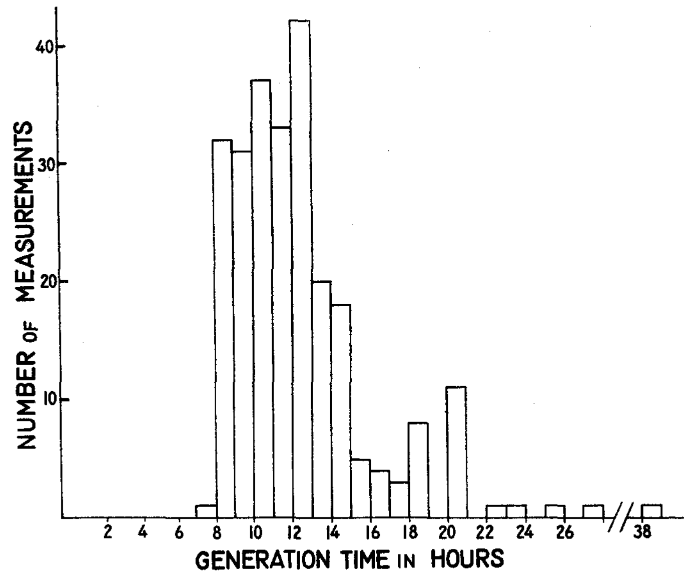

FIG. 3. Histogram to show the distribution of generation times.

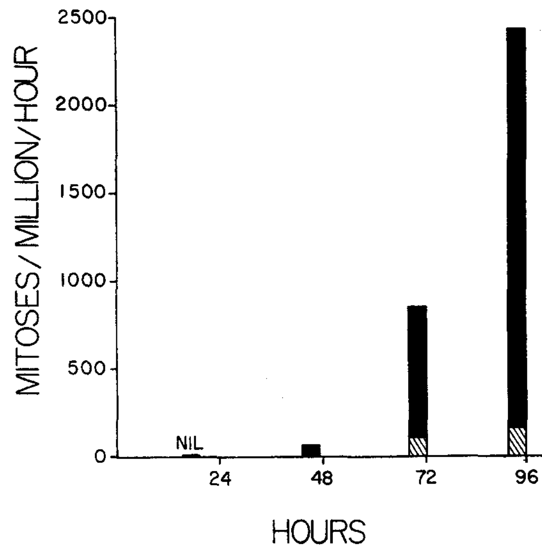

FIG. 4. Histogram to show the number of mitoses found in cultures of sensitive lymphocytes (Subject Sim) incubated with and without tuberculin. The solid parts of the bars represent mitoses in culture with tuberculin, the notched parts of the bars represent mitoses in control cultures without tuberculin.

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TABLE II

Mitosis Counts on Centrifuged Spreads after 4 hr Exposure to Vinblastine (Subject SIM)

	Culture without tuberculin				Culture with tuberculin			
Hr of incubation	22	46	72	94	22	46	72	94
No. of cells counted	9,491*	7,791	11,576	17,724	8,992*	11,251	8,313	14,086
Mitoses	0	0	5	12	0	3	28	136
Mitoses per million per hr	0	0	108	169	0	66	840	2,420

*These two counts were made on conventional spreads.

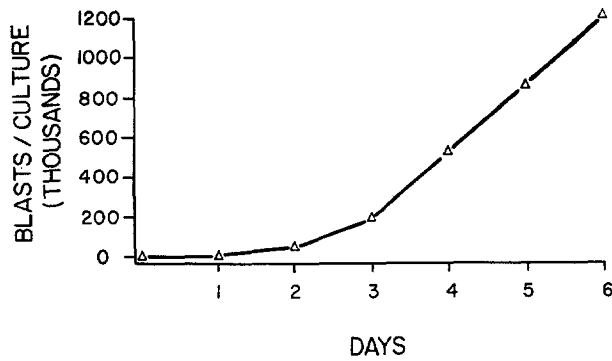

FIG. 5a

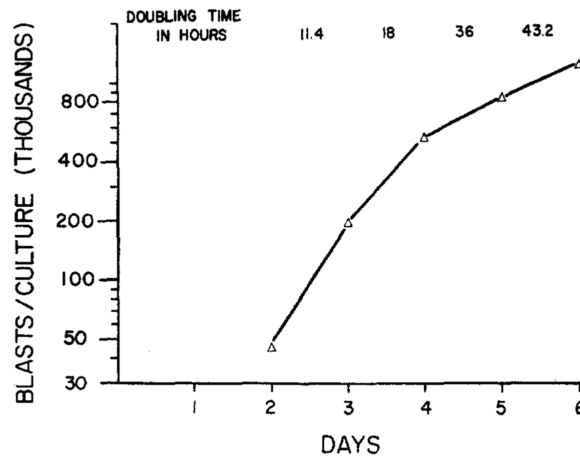

FIG. 5b

FIG. 5. Absolute counts of lymphoblasts in cultures of lymphocytes (Subject Cam) with tuberculin. No lymphoblasts were encountered at the start of the experiment nor after 24 hr incubation (Table III gives details). (*a*) Shows the counts displayed on an arithmetic scale. (*b*) Shows the counts displayed on a logarithmic scale; from this graph the doubling time for lymphoblasts during each 24 hr period has been calculated and the times are shown in hours.

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addition of antigen, with 66 mitoses/million per hr. By 72 hr there was a good mitotic response with 840 mitoses/million per hr.

Cell Counts.—Differential cell counts performed at daily intervals in a batch of tuberculin-stimulated cultures, obtained from another very sensitive individual (Cam), are given in Table III and are shown as graphs in Fig. 5. At the start of the experiment and after 24 hr incubation, no lymphoblasts were en-

TABLE III

*Differential Counts on Cells Cultured With and Without Tuberculin (Subject—Cam)**

Day	Total‡	Phagocytes and dead cells	Small lymphocytes	Intermediate forms	Lymphoblasts	Mitoses§
			Unstimulated cultures			
0	3500	1505	1995	—	—	—
1	3500	1575	1925	—	—	—
2	3480	1570	1910	—	—	—
3	3080	1600	1480	—	—	—
4	3120	1473	1647	—	—	—
5	2665	1060	1581	16	8	—
6	2355	1122	1172	61	—	—
			Cultures with tuberculin			
0	3500	1505	1995	—	—	—
1	3532	1592	1940	—	—	—
2	2980	1355	1550	30	45	—
3	2588	1088	1226	70	194	10
4	2824	815	1285	184	523	17
5	2880	645	1210	161	847	17
6	4005	1360	1124	321	1200	—

* Part of this data is shown as a graph in Fig. 5.

‡ Expressed as thousands per culture.

§ Refers to mitoses encountered during a routine differential count and is not a formal mitosis count.

countered. However, by 48 hr there were some 45,000 lymphoblasts in each tuberculin culture. This number represents 2.3% of the initial *lymphocyte* count and presumably the lymphoblasts arose by growth of small lymphocytes rather than by cell division. The doubling time for the lymphoblast population during successive 24 hr intervals after the 48 hr point can be measured from the semilogarithmic plot of counts (Fig. 5). Doubling times, thus, are 11.4 hr between the 2nd and 3rd days and then 18 hr, 36 hr, and 43.2 hr respectively.

DISCUSSION

These experiments performed on a captive lymphocyte population with time-lapse cinematography clearly show that repeated division of lymphoblasts occurred in antigen-stimulated cultures. Counts of mitotic figures, (allowing for

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possible inaccuracies; see methods) indicate that mitotic activity began about 48 hr after addition of antigen. This time is approximately the same as that observed in lymphocyte cultures stimulated by phytohemagglutinin (14). While some generation times were about 24 hr, most were much shorter—about 8–12 hr. This contrasts with earlier measurements on lymphocytes stimulated by phytohemagglutinin where interphase times of 24 hr and longer were found (10).

When events in microchambers and in standard tube cultures are compared in this way, the data suggest that clonal proliferation of the few lymphoblasts present at 48 hr could reasonably account for the much larger number of blasts which are present after 5–6 days incubation. If the lymphoblasts present at 48 hr developed into clones at the same rate as we have documented in single selected cells with time-lapse cinematography, and if all the lymphoblasts continued to divide, then this would account for a greater increase than that actually observed in test tube cultures, since the doubling time of the test tube population of lymphoblasts was in all instances longer than the generation times measured in single cells. Furthermore, the total increase in the lymphoblast population in tube cultures was between 16-fold and 32-fold; whereas single cells were sometimes observed to increase 64-fold. However, it must be emphasized that the mere observance of clonal proliferation does not exclude other postulated mechanisms (e.g., recruitment) that may contribute to an increase of the lymphoblast population in tube cultures. Under the conditions of these experiments, the possibility of recruitment occurring during the first 48 hr of test tube culture cannot be excluded and may indeed occur; preliminary evidence for such recruitment has been reported from this laboratory (15). The demonstrated ability of lymphoblasts to divide and redivide, nevertheless, does argue strongly for clonal proliferation as the major mechanism by which the cell population is increased after the first 2 days of culture.

The primary aim of these experiments, namely, to find out exactly how many cells are triggered into growth by contact with antigen, has not been reached. However, when the findings of clonal proliferation and of an onset to mitotic activity at 48 hr are coupled together, they point to the possibility of a very small number of reactive cells multiplying into a large population. The lymphoblast count at 48 hr in one experiment was 2.3% of the initial lymphocyte count; this is the nearest estimate we have made of this crucial number of cells. Our estimate is of the same order as that reported by Coulson & Chalmers (16) who found 17.8 blasts per cubic millimeter after 40 hr incubation, representing 0.9% of the starting lymphocyte population. Future experiments concentrating on the critical time period between 24 and 72 hr after the addition of antigen will help to evaluate the contribution, if any, of recruitment. Furthermore, accurate counts of mitoses at close intervals will be necessary to correlate the increase in cell numbers with the precise number of divisions observed.

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SUMMARY

When sensitive lymphocytes are cultured with the appropriate antigen, lymphoblasts appear after 24–48 hr of incubation and the number of these increases steadily from the 2nd to the 6th or 7th day. Our problem was to discover, at a cellular level, how this increase takes place; whether it is a massive response of many cells, stepwise recruitment of cells into the lymphoblast class, or simply repeated division of a few cells to form clones.

In these experiments lymphocytes were incubated with antigen in culture tubes for 2–4 days and then a few cells, usually less than 200, were transferred to special microchambers for further culture. In these microchambers the cells could be viewed continually with a microscope and their fate recorded over the next 3–5 days by time-lapse cinemicrography. Examination of the film produced in this way showed that lymphoblasts divided and redivided to produce clones of 64 cells or more. It was possible to measure generation times from the film for 301 cells; the majority were between 8 and 13 hr but the range was 7.5–38.0 hr. There was no clear difference between generation times of human lymphocytes stimulated with tuberculin, streptokinase-streptodornase, extract of the American pokeweed, or in the mixed leukocyte reaction. Similar times were also found for rat cells in the mixed leukocyte reaction.

While these observations show that clonal proliferation does occur and could reasonably account for all the increase of lymphoblasts in lymphocyte cultures, the experiments, because of their design, do not exclude the possibility that other mechanisms such as recruitment may play a role as well, particularly during the first 48 hr after contact between sensitive cells and antigens.

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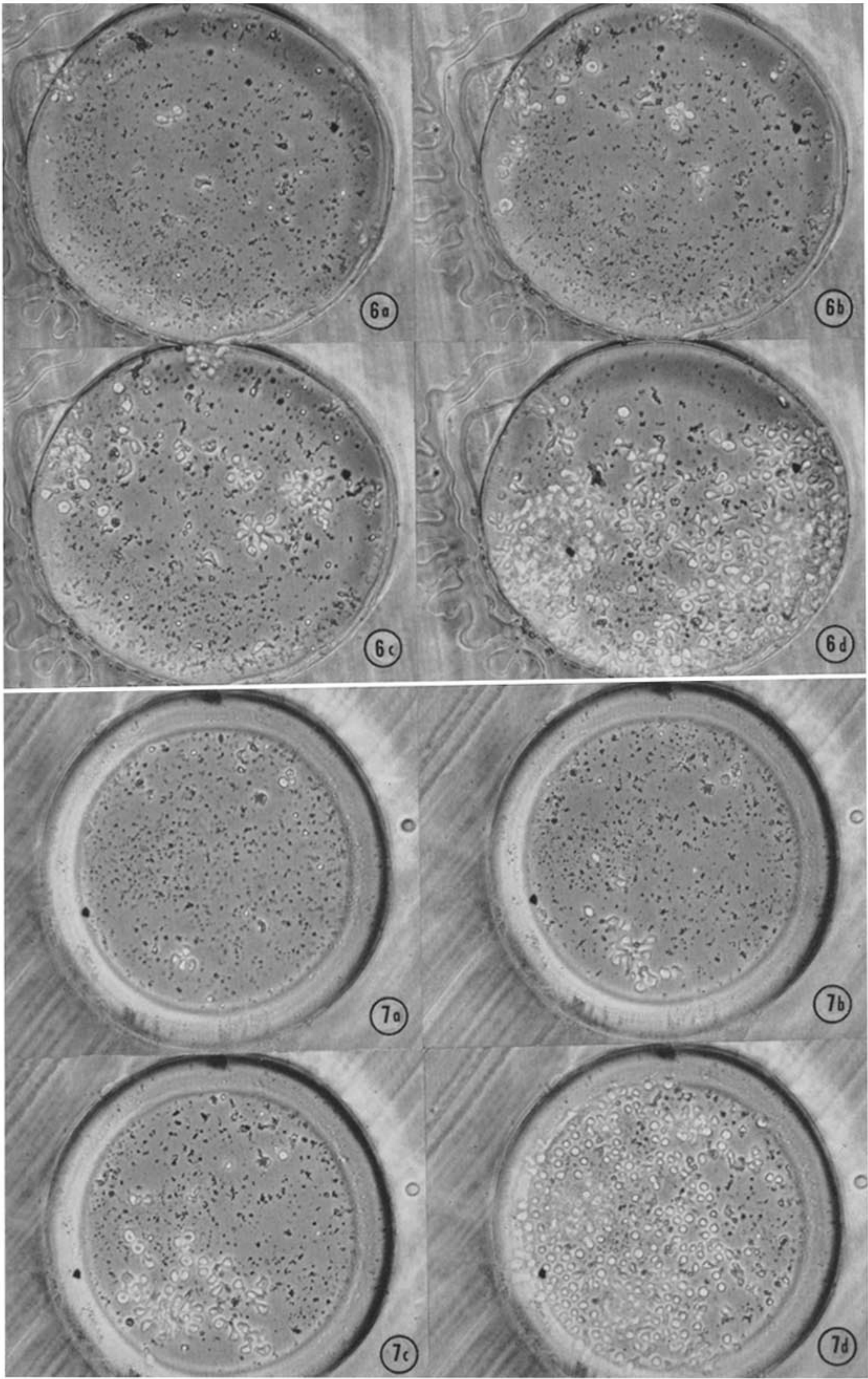
FIGS. 6 and 7. Two individual microchambers demonstrating tuberculin stimulated cell proliferation, photographed on *a*, day 3; *b*, day 4; *c*, day 5; *d*, day 6. The proliferating lymphoblasts are seen as large, light colored, refractile cells often connected in clusters. × 110.



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