CELL KINETIC STUDIES IN MIXED LEUKOCYTE CULTURES: AN IN VITRO MODEL OF HOMOGRAFT REACTIVITY*

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Abstract.—The recognition phase of homograft immunity can be studied in vitro in the mixed leukocyte culture reaction. In this reaction, at the end of seven days, up to 30 per cent of the lymphocytes in culture "respond" to a single allogeneic cell stimulus. This paper presents evidence that the lymphocytes responding in culture divide with the generation time of 18–21 hours, with some asynchrony, and that a large percentage of the cells found at the end of the culture period may be the products by division of a small number of cells initially responding. Two estimates are made of the frequency of the initially responding unit (the cell(s) responsible for initiating the response). These are probably minimum estimates and are in the range of 1 in 200 to 1 in 2000 cells which can initially respond. This very high frequency of responding units as compared with the responding units in immediate type hypersensitivity (antibody production) is discussed.

The in vitro mixed leukocyte culture (MLC) reaction[1, 2] probably represents the antigen recognition step of the homograft rejection response. In one-way MLC tests,[3] peripheral blood leukocytes (responding cells) of a potential recipient (A) interact with mitomycin C-treated lymphocytes (subscript m = stimulating cells) of a potential donor (B) in the mixed culture (AB_m). The lymphocytes (cells) of the recipient enlarge, incorporate thymidine, and divide in response to allogeneic (nonself) cells present in culture. Cells seem to respond only to antigens controlled by the major histocompatibility locus in man (HL—A).[4]

If cells of only one individual are incubated for seven days, more than 98 per cent of the small lymphocytes in culture remain small and do not incorporate thymidine; if cells of two unrelated individuals are mixed in an MLC test, up to 30 per cent or more of the cells in culture are enlarged and capable of incorporating thymidine at seven days. The present study was undertaken to study two possible explanations for this high percentage of cells responding to a single allogeneic cell stimulus: (1) that a large percentage of the cells (A) originally in culture respond to that allogeneic cell stimulus (e.g., B_m), and it is these cells which are finally enlarged, or (2) that a relatively small number of cells (A) initially respond, but that these cells divide repeatedly to give the final responding cell population.

To investigate the above possibilities, we measured the generation time of lymphocytes in MLC reactions, studied the rate of increase in the number of cells incorporating thymidine per 24-hour period from day 5 to day 8 of the reaction, and made two separate estimates of the frequency of initially responding units in MLC tests.

This paper presents evidence that cell division probably accounts for a major

part of the response seen in culture, and that the frequency of the initially responding unit is substantially higher than that found in the response of anti-body-producing cells to a single antigenic stimulus.[5, 6]

Methods.—One-way mixed leukocyte cultures: This method has been previously described.[3] In brief, heparinized blood is allowed to sediment, and the plasma is removed and centrifuged for 10 min at 150 g to obtain leukocytes. Cells to be used as "responding cells" are suspended in Eagle's minimal essential medium modified for suspension culture and supplemented to contain glutamine, penicillin, streptomycin, and 20% pooled cell-free plasma (MEM-S). Cells to be used as stimulating cells are suspended in 5–10 ml of MEM-S, incubated for 20 min at 37°C with mitomycin C at 25 μg/ml, twice washed in MEM-S, and resuspended in MEM-S. Responding cells are present in all cultures at final concentrations from 0.3 to 0.5 × 10⁶ mononuclear cells per milliliter; stimulating cells are present at concentrations of 0.5–0.8 × 10⁶ leukocytes per milliliter.[3] In some experiments, responding cell concentrations are reduced as described. Mixed cultures are distributed in 2.5-ml vol in glass tubes and incubated at 37°C in a humidified 4% CO_2 atmosphere for 7 days. After approximately 160 hr of incubation, 2 μc of tritiated thymidine (spec. act. 1.9 c per mmole) are added to each culture. Replicate cultures are harvested 5 hr later and acid-precipitable counts are measured. The results are expressed as counts per minute in each sample.

Harvesting for morphological studies: Colcemid is added to a culture, the cells of which had been earlier exposed to tritiated thymidine, for 2 hr, after which the cells are collected by centrifugation and suspended in approximately 0.5 ml of a hypotonic solution (0.075 mM KCl). Six minutes later, the cells are centrifuged and the sedimented cells are fixed in a solution consisting of three parts methanol and one part glacial acetic acid. The fixation procedure is repeated once more, after which the cells are recentrifuged and placed on glass slides. Slides are allowed to dry, after which they are stained with acetic orcein.

Autoradiography: Slides are dipped in Ilford nuclear research emulsion L4, exposed for 7 days, and then developed in D19 developer. Grain counts are performed with a 100× objective.

*Results.—*To determine the generation time of lymphocytes in culture, allogeneic cells were allowed to interact for four, five, or six days and were then labeled for one hour with tritiated thymidine. To halt the incorporation of labeled thymidine, a 1000-fold excess of nonradioactive thymidine was added to the culture in 0.1 ml normal saline. Cells were then harvested at intervals for autoradiography and the percentage of labeled cells and mitoses was recorded. Figure 1 shows the results of one such experiment in which the cells were labeled on day 5 of culture and followed for 39 hours. The solid line represents the average number of grains per labeled mitosis. At three hours after labeling, all mitoses examined were unlabeled; this suggests a G-2 period (the time between the end of DNA synthesis and mitosis) of greater than three hours. At 6 hours many mitoses were labeled, and by 9 and 12 hours, all mitoses were labeled. These are cells which were in the S period (time of DNA synthesis) during the initial labeling. With the elapse of more time, cells which were in G-1, M, and G-2 during the hour when radioactive thymidine was effectively present, and thus did not incorporate radioactive label, pass through mitosis; for example, at 21 hours none of the mitoses were labeled. By 33 hours, a second peak of labeled mitoses was present, with 80 per cent of mitoses labeled. Subsequently the percentage of labeled mitoses again decreased as the cells continued to cycle. The presence of approximately twice as many grains per labeled mitosis in the
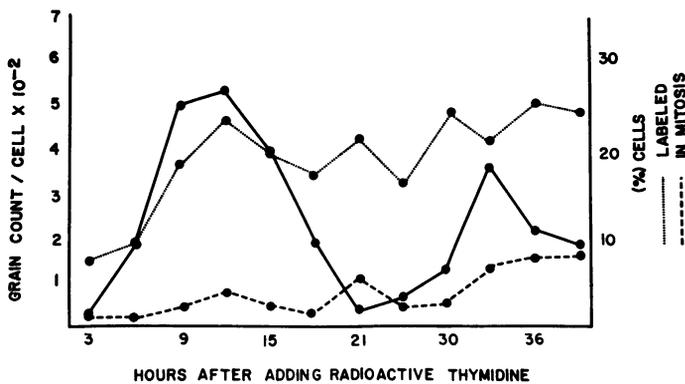
FIG. 1.—Mixed leukocyte cultures are labeled for 1 hr with tritiated thymidine. The solid line represents the average number of grains per mitosis at various times after labeling. The dotted line represents the percentage of labeled cells, and the broken line represents the percentage of mitoses.

first peak as in the second peak is substantiating evidence that these two peaks of labeled mitoses represented the same cell population dividing sequentially; at the peak of the first wave there were 535 grains per cell (average); at the peak of the second wave there were 355 grains. The highest number of grains in any one cell in the first wave was 1157; the second wave, 618. The dotted line represents the percentage of labeled cells in culture. Even at the beginning of the fifth day, 8 per cent of the cells in culture were labeled and during the next 36 hours this percentage increased to 28 per cent. The broken line represents the percentage of total mitoses in culture. At the beginning of the fifth day, 1 per cent of the cells were in mitosis, which percentage increased to 8 per cent by the end of this experiment.

In eight other experiments, including one in which cells of both individuals were responding, labeled at various times from day 4 to day 6, and then followed for periods from 36 to 57 hours, similar data were obtained: the generation time was between 18 and 21 hours, approximately half as many grains were found on the average in each succeeding peak of labeled mitoses, and in each case there was a steady increase in the percentage of labeled cells and mitoses. In one experiment the labeled cells were followed long enough to observe at 51 hours a third wave of labeled mitoses. Whereas in each of the nine experiments there was at least one time of sampling between the first and the second wave of labeled mitoses at which none of the mitoses were labeled (as at 21 hr in Fig. 1), this was not true after the second peak. After the second peak the percentage of labeled mitoses never dropped below 10 per cent, and seemed to level off between 10 to 30 per cent labeled mitoses in the five experiments terminated before 48 hours.

The percentage of labeled cells present on successive days gives some indication of the rate of increase in the number of dividing cells. However, if this is to give a measure of the percentage increase in responding cells, such numbers must be corrected for cell death, the number of nonlabeled cells in culture, and sampling errors of such techniques. A more accurate measure of the rate of increase in

the number of responding cells is obtained by measuring the incorporation of radioactive thymidine for a 5-hour period of labeling at 24-hour intervals on succeeding days. This provides an estimate of the number of cells passing through S. The results shown in Table 1 represent the average relative increases in thymidine incorporation for each successive 24-hour period. Thus on day 6 there is, on the average, a 2.9-fold greater incorporation of radioactive thymidine than on day 5, from day 6 to day 7 a 2.4-fold increase, and from day 7 to day 8 a 1.3-fold increase. The figures in the second column in the table give the 95 per cent confidence interval for these experiments and those in the third column give the highest increase found in any of the experiments for each particular 24-hour period. Since some of these mixtures gave maximum incorporation of thymidine on the sixth day, mixtures which showed a decrease between the sixth and the seventh day, or the seventh and the eighth day, are not included in the calculations. The peaks on days 6 and 7 probably account for the lower numbers for the period between the seventh and eighth days. Including only the data

TABLE 1. *Relative increase in thymidine incorporation in 24-hour periods.*

Day	Average	95 Per cent confidence interval	Maximum found
5–6	2.9	2.3–3.4	4.5
6–7	2.4	1.9–3.0	4.1
7–8	1.3	1.1–1.6	1.6

between day 5 and day 7, the average increase in any 24-hour period is 2.65-fold.

Two approaches were used to obtain an estimate of the frequency of the initially responding units in MLC tests. One of these methods has given a minimum estimate only; the other method gives an estimate of the frequency of dividing cells very early in culture.

One-way mixed leukocyte cultures were performed with decreasing concentrations of responding cells in the culture. Between 10 and 40 cultures were tested at each set of concentrations. We have previously shown[3, 7] that with increasing numbers of stimulating cells an increased response can be obtained using a constant number of responding cells, and that at some number of stimulating cells a maximum response is obtained. If concentrations of stimulating cells are raised still further, there is "less stimulation." For this reason each concentration of responding cells was tested at several concentrations of stimulating cells; all results given are those obtained at concentrations of stimulating cells that yield the maximum response obtainable at the concentrations of responding cells used. Results with twofold dilutions of responding cells tested at a single concentration of stimulating cells are given in Figure 2. The open bars represent control cultures (e.g., AA_m) and the solid bars allogeneic mixtures (e.g., AB_m). If we consider any allogeneic mixture that incorporates more than the mean counts per minute of the control cultures plus three standard deviations as a stimulating mixture, then with 12,500 responding cells, 100 per cent of allogeneic mixtures respond; with 6,250 responding cells, 100 per cent respond; with 3,125 responding cells, 100 per cent respond; with 1,563 responding cells, 65 per cent respond; with 782 responding cells, 55 per cent respond; and with 391 responding cells,
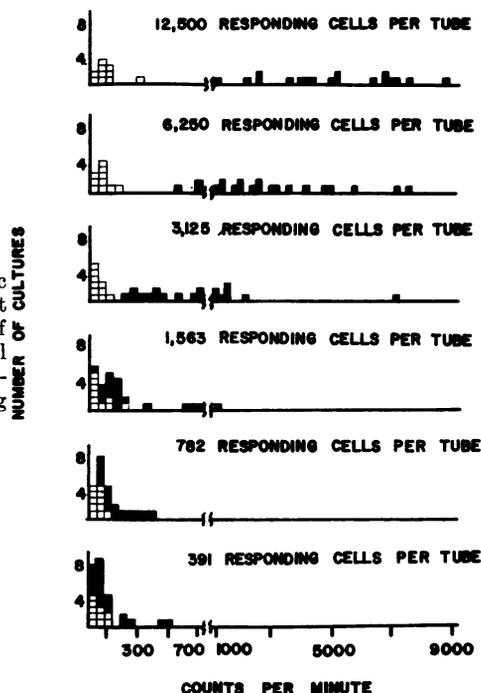
FIG. 2.—Solid bars represent allogeneic mixtures (e.g., AB_m); open bars represent controls (e.g., AA_m). The incorporation of radioactive thymidine in each of 10 control cultures and in each of 20 allogeneic mixtures, at varying concentrations of responding cells, is given.

25 per cent respond. In other experiments, approximately 50 per cent of allogeneic cell mixtures showed stimulation with numbers of responding cells ranging between 750 and 3,000 cells.

Since it is possible that not enough thymidine could be incorporated with such low numbers of responding cells to allow the detection of responding units, another approach was also tried. Cultures were set up at concentrations of stimulating and responding cells, as given in the methods described above, and on days 1, 2, 3, and 4 were examined by autoradiography for the frequency of cells incorporating radioactive thymidine. While this may not be an estimate of the frequency of the initial responding unit (which itself may not even divide), it does give an estimate of the frequency of cells which divide early in the culture period. The percentage of labeled cells in control cultures (AA_m) and in allogeneic mixtures (AB_m) was ascertained by autoradiography in six experiments. In a 5-hour period of labeling, between 16 and 21 hours after the cultures are set up, control cultures and allogeneic mixtures both show between 0/1,000 and 3/1,000 cells labeled. There was no significant difference between the cultures. Labeling between 40 and 45 hours after the beginning of culture does show a difference in some experiments. The net number of labeled cells in the allogeneic mixtures increased from an average of approximately 5/1,000 labeled cells on the second day to approximately 20/1,000 labeled cells on the fourth day.

Discussion.—These experiments present evidence that lymphocytes in mixed leukocyte cultures divide with a generation time of 18–21 hours. Marshall *et al.*,[8] by direct observation, found that the average time of cell division in their

system was approximately 12–13 hours, with some cells in the cultures dividing
with generation times of more than 20 hours.   (Differences in technique could
account for variation in the division time, rapidity with which the reaction is
initiated, etc.)   Our experiments would agree with their findings that there
must be some asynchrony in the dividing cell population.   The majority of cells
in our system were probably dividing with the generation time presented above,
and thus there is a period between the first and the second peak of labeled
mitoses when there are no labeled mitoses present.   The presence of 10–30 per
cent unlabeled mitoses in the second peak, with 70 per cent unlabeled mitoses in
the third peak, observed in one experiment, and the failure to find a time-point
when all mitoses are unlabeled after the second peak of mitoses could be accoun-
ted for either by asynchrony of the type suggested above or by recruitment of
new cells into the dividing cell population.   With the above generation time, we
could assume, given that every cell that divides once keeps dividing (consistent
with the findings of Marshall *et al.* that one cell divides eight times), that in a
24-hour period there could be an increase between 2.3- and 2.7-fold in the number
of cells dividing.

Table 1 presents data which suggest that the actual increase in the dividing
cell population is in the range mentioned above.   It must be stressed that this
only means that the increase *could* be accounted for by cell division, and does not
mean that other factors such as recruitment, mentioned above, do not take place.
Indeed, there are examples where the increase found in a 24-hour period (3rd
column, Table 2) could not be accounted for solely by cell division.   This may
however, reflect technical variation.

The attempts to determine the frequency of a responding unit in mixed
leukocyte cultures by diluting the responding cells to a point where the frequency
of a responding unit would fit the Poisson distribution only yielded a minimum
estimate.   The assay of thymidine incorporation may not be sensitive enough to
differentiate, in cultures containing fewer cells than those tried, between the
failure to respond, reflecting the absence of a responding unit, and the failure of
responding cells to incorporate enough thymidine to allow detection.   In fact,
the percentage of allogeneic mixtures showing significant stimulation does not, in
most cases, decrease logarithmically as would be expected if the number of stimu-
lating cultures (at a given total cell number) followed a Poisson distribution.
This is probably due to the high frequency of responding units in each culture;
lower numbers of cells are needed to keep the maximum number of responding
units at one per culture.   One responding unit per 1,500–2,000 cells is thus a
minimum estimate.

The study of the frequency of dividing cells early in culture may be a direct
measure of the frequency of the responding unit if the initially responding cell is
a dividing cell and subsequent dividing cells are progeny of that first cell.   If
however, the responding unit is a cell which does divide but which can recruit
other cells to enter the response, the estimate of the number of cells "dividing"
in culture is probably a high one with respect to the actual responding unit.   The
data presented can be interpreted in at least two ways.   First, one can assume
that under these conditions there is a lag period of several hours before the re-

sponse gets under way and, basically, the first responding cells do not divide until the second day. Given this assumption, we would estimate that between 1/200 and 1/500 cells initially divide. Second, we could assume that the response on the first day involves too few cells to differentiate the number of cells incorporating thymidine in the allogeneic mixture from the isogeneic control and we should extrapolate the curve obtained for days 2, 3, and 4 back to day 1. If we do this, the frequency of initially dividing cells will be lower than that given above.

Combining our data obtained with the two approaches, we can say that the minimum frequency of the initially responding unit is between 1/200 and 1/2,000 cells present in culture. This estimate is in general agreement with those made by others.[9-11] One possible reason for a higher frequency of responding cells in the experiments of Simonsen[12] is that in MLC tests only major locus antigens are recognized, whereas cells mediating graft versus host reactions may be able to recognize antigens determined by minor loci as well.

It is possible to make a calculation to see whether the proposed generation time could account for the total number of cells responding at seven days. Any such calculation must make many assumptions and is at best an approximation. Since the total number of lymphocytes in a mixed leukocyte culture usually does not vary by more than a factor of 2 during the seven days of incubation, let us ignore cell death. At the beginning of culture there are 7.5×10^5 responding lymphocytes present in culture. At the end of culture, assuming a similar number of cells present, 2×10^5 lymphocytes may be enlarged and incorporating thymidine. If the frequency of the initial responding unit is 1/1,000 and the division time is 20 hours, then we have 75 responding units that can undergo 8.5 divisions each. Assuming that every cell that divides once continues to divide, this would yield $75 \times 2^{8.5}$ cells responding. Although no conclusions can be reached from any such calculation, the number of responding cells that might be accounted for by cell division alone is clearly appreciable.

There are three possible explanations for the very high frequency of responding units in the homograft recognition response as compared with antigen recognition in immediate-type hypersensitivity. In the latter reaction the estimate is that there is only one responding unit per million cells.[5, 6] First, in the homograft system we are dealing with a single locus in MLC tests, each allele of which can determine many different antigenic specificities. It is impossible to estimate how many different "haptenic" specificities could be associated with a single allele; however, this number could certainly be greater than 10. The frequency of responding units must be interpreted as the frequency of units that can respond to any one or more of these specificities; the frequency of a responding unit that could respond to only one of the specificities would be lower by some factor. Second, the possibility that cells responding to the histocompatibility antigens are pluripotential must be considered. We shall elsewhere present evidence that the cells which respond to one set of allogeneic cells may be in part the same cells that respond to a second or even a third set of allogeneic cells. While this evidence can be interpreted in terms of sharing or cross-reactivity between the antigens present on different allogeneic-stimulating cells, which is known to be true, it can also be interpreted as a reflection of pluripotentiality of responding

cells.   Third, it is conceivable, although not attractive in present terms, that recognition in cellular immune reactions is not mediated by antibodies but by a completely different type of recognition phenomenon.

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