THE ACTIVATION AND REACTIVATION OF PERIPHERAL LYMPHOCYTES IN CULTURE

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SUMMARY

Human lymphocytes stimulated for 16 h and then cultured without stimulant showed maximal activity on days 2-3 following a stimulus of phytohaemagglutinin (PHA) and on days 3-4 following a stimulus of staphylococcal filtrate (SF). At low dosage of stimulant the response of the cells was less marked but persisted for a longer period than at high dosage. The pattern of response is discussed in relation to the mechanism of activation.

After the effect of the initial stimulus had died away cell populations which had been stimulated with SF or PHA could be restimulated with either stimulant. Their response, when stimulated this second time, was quicker than that of incubated cells from the same donor which had not been previously stimulated. Prestimulated cells were also tested in two immunospecific reactions: the reaction to tuberculin-purified protein derivative (PPD), and the mixed lymphocyte reaction. Cells which had been previously exposed to SF responded more quickly to PPD than cells not previously stimulated. Cell populations which had been previously stimulated also reacted more quickly in a mixed lymphocyte reaction. It is concluded that lymphocyte populations which have been recently stimulated not only retain their capacity to react to immunospecific mitotic stimuli but also react more quickly.

INTRODUCTION

The peripheral lymphocytes of many species are activated to undergo blast formation by PHA and SF (Knight, Ling, Sell & Oxnard, 1965). The mechanism of this activation is unknown. In the case of human lymphocytes a high proportion of the lymphocytes of most individuals will, under the right conditions, respond to these stimulants (Ling, Spicer, James & Williamson, 1965). A superficially similar activation, of lower degree, is produced by culturing lymphocytes from tuberculin-positive but not tuberculin-negative persons with tuberculin-purified protein derivative (Pearmain, Lycette & Fitzgerald, 1963; Cowling, Quaglino & Davidson, 1963; Aspergren & Rorsman, 1964). There is evidence that the different reactivity of the lymphocytes from the two types of donor is not due primarily to an inherent difference in the cell responsiveness of the individuals concerned but is determined by the donor's previous exposure, or lack of exposure, to the tubercle antigens. The reaction is thus thought to be a 'recognition reaction' in vitro of antigens previously encountered in vivo. It resembles the specific response to antigen in vitro of the spleen cells from primed rabbits reported by Dutton & Eady (1964).

Even with stimuli which activate a high proportion of the lymphocytes of most individuals and animals such as phytohaemagglutinin and staphylococcal filtrate there is a
considerable variation in cellular responsiveness in relation to dose of stimulant and the time of incubation required. When lymphocyte populations show a relatively big response to a specific stimulus, such as tuberculin, the heightened reactivity may be latent in the whole population of lymphocytes from that donor or it may reside in only a small proportion of the population. The reactivity of the individual lymphocytes may also be governed by other characteristics of the cells such as their age and the presence or absence of other cells such as macrophages and polymorphonuclear leucocytes.

Bain, Vas & Lowenstein (1964) have shown that blast cells appear, after several days, in cultures of mixed lymphocytes from two unrelated individuals. The degree of inter-stimulation which arises from lymphocyte–lymphocyte interaction is related to the genetic disparity of the cell donors (Bain & Lowenstein, 1964). Considerable attention has been paid to this reaction as a potential histocompatibility test. It has also been shown that material active in stimulating homologous cells is shed off into the medium by the cultured leucocytes (Gordon & MacLean, 1965; Kasakura & Lowenstein, 1965).

In the work reported here human or rabbit peripheral lymphocytes have been incubated with a stimulant for a short period, the stimulant having been removed before DNA synthesis has begun. The cells have then been incubated for a longer period and, after recovery from the first stimulus, have been subjected to a second stimulus of the same or of a different kind and their response compared with that of cells not previously exposed to a stimulant. The behaviour of stimulated cells and their progeny in the mixed lymphocyte reaction has also been examined.

MATERIALS AND METHODS

Blood lymphocytes

These were obtained by a method similar to that of Coulson & Chalmers (1964). Human venous blood or rabbit blood, obtained from the marginal ear vein, was defibrinated, mixed with 3% sterile gelatin (fine grain pigskin gelatin, British Glue and Chemicals Ltd.) in saline and allowed to stand for 1–2 h at 37°C to sediment the erythrocytes. The leucocyte-rich serum was removed and the leucocytes counted. The cells were spun down by low-speed centrifugation in sterile, capped polycarbonate tubes and resuspended in their own serum-gelatin to a leucocyte concentration of $6 \times 10^6$/ml.

Lymphocyte cultures

In bijoux. Bijoux (volume 7 ml) contained leucocyte suspension (0.5 ml), stimulant (when added) and Eagle's medium to a volume of 3 ml.

In tubes. The second stage of most experiments was performed in tubes rather than bijoux because larger numbers of cultures could be set up with a given number of cells while maintaining unaltered the cell concentration and depth of fluid. Test-tubes (3 x 4 in.) contained leucocytes suspended in 1 ml of Eagle's medium containing 20% of homologous serum-gelatin and loosely fitting aluminium caps were applied. The cell concentration was usually $1-2 \times 10^6$/ml for human lymphocytes and $2 \times 10^6$/ml
for rabbit lymphocytes. Bijoux and tubes were placed in an air-tight jar (desiccator) and gassed with 5% CO₂ in air.

**Combined.** The following procedure, which was employed in an experiment using stimulated and unstimulated mixed human lymphocytes, will serve to illustrate the technique. Blood (50 ml) from each of 2 individuals (L. and W.) was defibrinated and mixed with 3% gelatin in saline (17 ml to each blood sample) and allowed to sediment for 1 h. The leucocyte-rich serum of L. (volume 40 ml) contained \(1.5 \times 10^6\) w.b.c./ml and of W. (48 ml), \(1.1 \times 10^6\) w.b.c./ml; the cells were spun down and resuspended in 10.0 ml and in 8.0 ml, respectively, of L. serum. L. serum was regularly used for the suspension of cells in experiments of this kind as it was found that the degree of stimulation depended on the serum used (Holt & Ling, unpublished observations). Aliquots of the cell suspensions (0.5 ml) were distributed into bijoux (20 of L. and 16 of W.); SF (1.0 ml) was added to half of the bijoux in each case and the volumes of all the bijoux were made up to 3 ml. The bijoux (caps loose) were gassed with 5% CO₂ in air and incubated at 37 °C. After 16 h four pools of the cells were made: L. stimulated, L. unstimulated; W. stimulated, and W. unstimulated. The w.b.c. totals for each pool were 15, 20, 15 and 13 x 10⁶ respectively. The cells were spun down, the supernates completely removed and the cells resuspended in 15, 20, 15 and 13 ml respectively of Eagle's medium containing 20% of L. serum-gelatin (saved from the previous day and stored at 4 °C). Six rows of 8 tubes were set up in a rack. The leucocyte suspensions were added to the rows of tubes so that their contents were: 1st row, stimulated L. cells (1 ml); 2nd, stimulated W. cells (1 ml); 3rd, stimulated L. plus stimulated W. cells (0.5 ml+0.5 ml); 4th, unstimulated L. cells (1 ml); 5th, unstimulated W. cells (1 ml); and the 6th, unstimulated L. cells plus unstimulated W. cells (0.5 ml+0.5 ml). The capped tubes were gassed in 5% CO₂ in air at 37 °C. \[^{14}C\]Thymidine (0.12 µc) was added at intervals to the series of tubes as a standard drop (0.03 ml) and the tubes gently agitated. When tubes were removed the remainder were regassed. The day indicated in the various Figures refers to the day of harvesting.

Eagle's medium was prepared in the department of Virology and Bacteriology, University of Birmingham. It contained 10% (v/v) tryptose broth and penicillin and streptomycin.

Staphylococcal filtrate (SF) was the Seitz filtrate from Micrococcus pyogenes var. aureus grown for 5 days in Parker 199 medium (Ling et al. 1965). It was active against human and rabbit lymphocytes. In restimulation experiments it was used at a final dilution of 1 in 3 except where otherwise stated.

Tuberculin-purified protein derivative (PPD) was an acetone-ether dried preparation (PPD 10) of human tubercle bacillus obtained from Dr I. Lesslie of the Central Veterinary Laboratory, Weybridge. It was only partially soluble in saline and was used at a concentration of 10 or 20 µg/ml.

Phytohaemagglutinin (PHA) was a purified preparation of PHA (batch x 5) obtained from Dr B. A. L. Hurn of Burroughs Wellcome and Co. When used at a final concentration of 0.5 µg/ml it was found to be a very potent activator of all samples of human lymphocytes tested. Surprisingly, it produced very little activation of rabbit lymphocytes when tested under the same conditions.
Thymidine labelled with $^{14}$C or $^3$H ($^{14}$C-TdR or $^3$H-TdR) was obtained from the Radiochemical Centre, Amersham. $[^{14}$C] Thymidine was of specific activity 36 mc/mM. It was diluted to a concentration of 0.12 $\mu$g/ml in 0.03 ml. Thymidine-(methyl-$T$) was supplied at a specific activity of 3000 mc/mM. This solution was treated with 'cold' thymidine before addition to cultures, usually to a specific activity of 6 mc/mM. It was diluted to a concentration of 1 $\mu$g/ml. The thymidine ($^{14}$C-TdR or $^3$H-TdR) was usually added to cultures 16 h before harvesting the cells.

**Scintillation counting and autoradiographs**

Cultures to which $^{14}$C-TdR had been added were centrifuged and the cell pellet washed with saline. The cells were suspended in saline (1 ml) and trichloroacetic acid (1 ml of 10 %) added. The suspension was centrifuged and the pellet washed with methanol and finally suspended in 1 ml of methanol and washed into the counting bottle with 10 ml of scintillation fluid (xylene containing 0.6% P.P.O. and 0.012% P.O.P.O.P.) which had been diluted 2 in 3 v/v with Triton X-100 (Lennig Chemical Co., London, W.C. 1) as recommended by Patterson & Green (1965). The radioactivity in the suspension was assayed in a Packard Tri-Carb automatic scintillation counter with an efficiency of approximately 66%. The counts were corrected for chemical quenching by the channel-ratio technique.

Autoradiographs of cells incubated with $^3$H-TdR were made with AR 10 stripping film following the technique of Doniach & Pelc (1950). The autoradiographs were developed at 4 °C for periods of 1 week to 3 months and stained through the gelatin by the method of Gude, Upton & Odell (1955).

**EXPERIMENTAL RESULTS**

Response of lymphocytes treated for short periods with SF or PHA. Culture of rabbit or human lymphocytes in the presence of staphylococcal filtrate resulted in activation of a high proportion of the starting population of small lymphocytes into blast cells with a lag period of about 24 h before DNA synthesis began. Mitotic cells were observed on the 3rd, 4th and 5th days. When the cells were exposed to the stimulant for 16 h instead of the whole culture period similar or higher levels of $[^{14}$C] thymidine incorporation by the cells were registered and there was no difference in the pattern of response until after the 4th day by which time many of the activated cells had completed one cell cycle (Fig. 1(a)). A marked increase in the number of blast-like cells occurred before an appreciable number of thymidine-labelled cells appeared (Fig. 1(b)).

Batches of human leucocytes were subdivided and exposed to a range of doses of SF for 16 h and then cultured in the absence of stimulant. All doses produced a maximal response on day 4. It was evident that higher doses produced a more rapid rise and fall in the rate of thymidine incorporation; a lower dose produced a slower rise and a more sustained level of thymidine incorporation (Fig. 2). The difference between the curves could not be accounted for by a difference in survival rate of the cells.

Similar experiments using a 16-h pulse of PHA at various dosages showed the same
range of effects as with SF but with some points of difference (Fig. 3). The peak activity when moderate or high doses of PHA were used occurred on day 3, instead of day 4. With a low dose of PHA, on the other hand, thymidine incorporation was spread over days 3, 4 and 5. The activity of cultures containing no added stimulant remained low until day 4 and then slowly increased, often reaching appreciable levels between days 6 and 9.

Fig. 1.
(a) [14C]Thymidine incorporation of rabbit peripheral lymphocytes stimulated with SF. All cultures initially contained, per ml, 6 x 10^6 leucocytes, 0.17 ml SF, 0.3 ml rabbit serum, and 0.53 ml Eagle’s medium. After 16 h the cells were spun down and resuspended to a cell concentration of 2 x 10^6 per ml in a medium of the same composition with or without the omission of SF. Unbroken line, SF omitted; broken line, SF present.
(b) Blast formation in cultures of rabbit lymphocytes which had received a 16-h pulse of SF. Line with points, blasts labelled with [3H]thymidine; line with crosses, unlabelled blasts.
(c) Restimulation with SF of rabbit lymphocytes which had been exposed to a 16-h pulse of SF. Line with points, [14C]thymidine incorporation of cells which received a first and a second stimulus; line with points, [3H]thymidine incorporation of cells which received the first stimulus only.

Progeny of activated cells. Rabbit lymphocytes were exposed to SF for 16 h and grown for a further 24 h in the absence of stimulant; then [3H]thymidine (1 μCi/3 ml of culture) was added for the ensuing 24 h and the cells were again washed and cultured for a further 24 h in Eagle’s medium + rabbit serum containing 50 μg/ml of ‘cold’ TdR, when smears were made and autoradiographs prepared. Some of the labelled cells were classified as typical blasts, some as typical small lymphocytes (see Figs. 9–12) but the majority were intermediate between these two extremes. Progeny resembling small lymphocytes were less common in cultures of human peripheral cells.

Restimulation of lymphocytes. When rabbit or human cells which had returned to resting levels of activity after a pulse stimulation with SF or PHA were re-exposed to
the same stimulants a pronounced further incorporation of $[^3H]$thymidine was obtained (Fig. 1(c) and Table 1). It appeared to be immaterial whether the first and second stimuli were identical; populations of lymphocytes which had been treated with either stimulant were more responsive than untreated lymphocytes. This phenomenon was investigated in more detail by testing cultures at different times after the second stimulus. It was found that prestimulated lymphocytes responded more quickly to the second stimulus than cells not previously exposed to stimulant (Fig. 4), and the response was over more quickly; the maximum of the response was slightly higher on

![Graph](Image)

**Fig. 2.** $[^{14}C]$Thymidine incorporation of human lymphocytes exposed to SF (0, 0.5, 1.0, or 2.0 ml/3 ml culture) for 16 h and cultured subsequently in the absence of stimulant. Initially each of the four batches of cells contained $1.4 \times 10^8$ leucocytes per ml. After the 16 h exposure to SF the cell counts were $0.9$, $0.8$, $0.9$, and $0.8 \times 10^8$ per ml, respectively. The cells in each batch were spun down, the supernates discarded and the cells resuspended to a cell concentration of $1.3 \times 10^8$ per ml in Eagle’s medium + serum and divided into aliquots for further culture.

some occasions and lower on others. Much the same picture emerged when similar cultures were examined morphologically. Blasts in DNA synthesis appeared earlier and in greater numbers in the restimulated cultures. It was further found (as might be expected from an examination of Figs. 3 and 9–12) that populations of lymphocytes stimulated once with SF and then cultured for a longer period without stimulant frequently reverted to a lower degree of activity in terms of thymidine incorporation than cells not subjected to stimulation. The thymidine incorporation of unstimulated lymphocytes usually began to rise appreciably on day 6 and had reached quite a high level by day 9.

**Effect of prestimulation on the response to PPD.** Cells exposed to PPD for 16 h and washed still had a high level of DNA synthesis after a further 4 days of culture. This
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Fig. 3. [14C]Thymidine incorporation of human lymphocytes exposed to PHA (final concentration 0.25, 0.5, 1.0, or 2.0 μg/ml) for 16 h and cultured subsequently in the absence of stimulant. Initially each of the five batches of cells contained \(1.3 \times 10^8\) leucocytes per ml. After the 16-h exposure to PHA the cell counts were 0.8, 0.7, 0.8, 0.6 and 0.6 \(\times 10^6\) per ml, respectively. The cells were then spun down, the supernates discarded and the cells resuspended to a cell concentration of \(1 \times 10^6\) per ml in Eagle's medium + serum and divided into aliquots for further culture.

Table 1. Restimulation of human lymphocytes

<table>
<thead>
<tr>
<th>Stimulant used</th>
<th>During the first 16 h</th>
<th>From 96 h onwards</th>
<th>[14C]Thymidine uptake during the period 120–144 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(counts per min)</td>
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<td></td>
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<td>(2)</td>
</tr>
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<td>3221</td>
</tr>
<tr>
<td>PHA</td>
<td>PHA</td>
<td>13350</td>
<td>15650</td>
</tr>
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</table>

Columns (1) and (2) show results with two different batches of human lymphocytes (1.5 \(\times 10^8\) ml cells per culture). PHA concentration, 0.5 μg/ml; SF concentration, 1 in 6.
Fig. 4. Restimulation of human lymphocytes with SF. The two graphs depict the results of two separate experiments. In each case half of the peripheral lymphocytes from a human donor were exposed to SF (1 in 3) for 16 h; the other half were incubated for the same time without stimulant. The cell concentrations at the beginning of the culture were $2 \times 10^6$ per ml. For the period up to the second stimulus both populations were cultured without stimulant. At the end of this period the cell concentrations were $0.85$ and $0.80 \times 10^6$ per ml (stimulated) and $0.95$ and $0.90 \times 10^6$ per ml (unstimulated). At the time of the second stimulus half of the cells from each lot were exposed to SF (1 in 3); the other half were not stimulated. The cell concentration in all tubes was standardized at $2 \times 10^6$ per ml. Unbroken lines, cells which received the first stimulus; broken lines, cells which did not. The upper lines on both graphs refer to cells which received the second stimulus; the lower lines to cells which did not.

Table 2. Stimulation of human lymphocytes with SF and PPD

<table>
<thead>
<tr>
<th>Stimulant used</th>
<th>During the first 16 h</th>
<th>From 96 h onwards</th>
<th>Days 4-5</th>
<th>Days 5-6</th>
<th>Days 6-7</th>
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</thead>
<tbody>
<tr>
<td>PPD</td>
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<td></td>
<td>10800</td>
<td>14200</td>
<td>11300</td>
</tr>
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<td>4380</td>
</tr>
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<td>5080</td>
<td>11790</td>
<td>19490</td>
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<tr>
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<td>PPD</td>
<td></td>
<td>1800</td>
<td>2950</td>
<td>11680</td>
</tr>
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</table>

was probably due to the fact that the PPD was not completely soluble so that stimulant was not completely removed from the cultures and spontaneous restimulation occurred, giving rise to a peak at the same point as in the culture to which further PPD had been added (Table 2). The reverse experiment gave a clear-cut result. Cells previously cultured with SF responded more quickly to a subsequent stimulus of PPD than cells incubated without stimulant (Fig. 5).
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Effect of prestimulation on the mixed lymphocyte reaction. Mixtures of freshly separated leucocytes from any two unrelated individuals always showed evidence of interstimulation. The differential thymidine incorporation of the mixtures rose steadily, reaching a peak on day 6. Leucocytes which had been incubated in vitro in their own serum plus Eagle's medium for several days showed evidence of interstimulation more quickly after mixing than mixtures of fresh cells but the increase in thymidine incorporation resulting from the mixing never reached the maximum achieved with equivalent numbers of mixed fresh cells (Fig. 6). Lymphocytes which had been pre-stimulated with SF interacted after mixing more quickly than the corresponding incubated cells, but they reached a roughly equivalent maximum of thymidine incorporation (see Fig. 7). In order to ascertain whether the presence of large numbers of active blasts in the mixture affect the rate or the course of the mixed cell reaction some batches of leucocytes were stimulated by incubation with SF for 16 h and then mixed without delay. It will be seen from Fig. 8 that the degree of interaction of the two preparations, as judged by their incorporation of [3H]thymidine, was unaffected by preactivation. A difference emerged after day 6 when the increment of activity due to mixing of prestimulated cells rapidly declined in comparison with that of unstimulated cells.

Effect of pre-immunization of rabbits on the reactivity of their lymphocytes in culture. The peripheral lymphocytes of 4 rabbits which had been immunized with sheep erythrocytes in Freund's adjuvant gave a response to SF of approximately the same magnitude and time scale as those of normal animals.
Fig. 6. The mixed lymphocyte increment of fresh and incubated human lymphocytes. Cells were incubated for 4 or 6 days or mixed immediately as indicated. The increment due to mixing was obtained by subtracting half the sum of the activity (expressed as counts/min $[^{14}C]$thymidine incorporated into the cells) of cells cultured separately from the activity of mixed cell cultures at the same cell concentration.

**DISCUSSION**

Some of the properties of lymphocytes exposed to stimulants described in this paper parallel those of chick embryo fibroblasts exposed to embryo juice (see review by Swann, 1958). Jacoby, Trowell & Willmer (1937) found that embryo juice need only be added to chick fibroblasts for 1 h or less to produce a crop of mitoses 10–20 h later. Further addition of embryo juice had no effect until some 16 h after the initial addition when it would produce a crop of mitoses starting after a further 10 h by acting on cells which had just completed a mitosis. Five per cent of juice activated a few cells but left the majority unaffected; 15% activated appreciably more and 40% more again. A second application of juice at the same strength after the first crop of mitoses was over produced a similar effect on the progeny of the cells originally
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Fig. 7. The effect of prestimulation with SF on the human mixed lymphocyte reaction. Unbroken line, prestimulated cells; broken line, incubated unstimulated cells. The counts/min represent $[^{14}C]$thymidine incorporation by the mixtures after correction for the activity in cultures of cells from the two donors unmixed. The initial cell concentrations were $2 \times 10^6$ per ml and the cells were resuspended to this concentration for the mixed cell reaction.

Fig. 8. A comparison of the degree of interaction of activated human lymphocytes and unstimulated human lymphocytes. One half of the lymphocytes from each of 2 donors was incubated with SF for 16 h before mixing; the other half was incubated without stimulant for the same period before mixing. Unbroken line, increment due to mixing of stimulated lymphocytes; broken line, increment due to mixing of unstimulated lymphocytes.
activated while those cells originally unaffected seemed to remain unaffected. There are obvious points of resemblance here to the response of lymphocytes to stimulants, but in applying this sort of reasoning to lymphocytes we have to take into account the additional factor of stimulant specificity. A population of lymphocytes from a given individual may be responsive to both SF and PPD although the actual cells which are stimulated, or more easily stimulated, by each preparation might be different. Primary stimulation with SF, by altering the ratio of stimulant-specific cells in the culture, might then be expected to enhance the effect of a second dose of SF applied later but to reduce the response to PPD. If, on the other hand, the same cells were triggered off equally by both stimulants, primary stimulation with SF might be expected to enhance the subsequent response to either stimulant. The second type of effect was regularly observed, suggesting that the same lymphocytes are responding to either stimulant.

Lymphocyte stimulants, like the embryo juice on fibroblasts, exhibit a dose effect. The population of lymphocytes activated by a given level of stimulant appears to be unaffected by the same stimulant until the cell cycle has been completed and given rise to progeny which are susceptible to stimulation. The insusceptibility of the activated lymphocytes during this period does not necessarily extend to other stimuli; for instance, stimuli to differentiate into antibody-forming cells, but the fact that the cells retain their potential for response to immunospecific mitogenic stimuli after exposure to a different mitogenic agent makes it unlikely that differentiation has occurred.

Cells stimulated with PHA or SF respond more readily to either stimulus than unstimulated cells. More significantly, lymphocyte populations from tuberculin-positive individuals respond more readily to tuberculin PPD after stimulation with SF than lymphocytes from the same individual incubated without stimulant. In a similar manner evidence of interstimulation of two mixed populations of lymphocytes appeared more quickly when some of the cells had recently been induced to divide by stimulation with SF or PHA. These results must be interpreted as meaning, first, that the capacity for specific reactivity is a built-in property of the cells and is not modified quantitatively by the stimulant to which they are exposed, and secondly, that recently stimulated populations are hyper-reactive to further stimuli. The activation process does not appear to follow an identical course with all stimulants since the peak period of thymidine incorporation for moderate or high doses of stimulant is variable but characteristic for that stimulant. The slow rise in DNA synthesis which occurs after about 7 days in cultures not deliberately stimulated is presumably due to a very low level of stimulation by traces of substances (perhaps bacterial) accidentally introduced during handling or by the antibiotics present (Johnson & Russell, 1965).

It cannot be said with certainty that the progeny of pulse-stimulated lymphocytes become exactly like small lymphocytes again after a recovery period, but in our cultures the progeny of some of the cells labelled during the synthetic period were quite indistinguishable in stained smears from small lymphocytes. The cells which had recovered from a pulse stimulus were also just as capable of responding to a different stimulus as the untreated lymphocytes. The only difference in behaviour which could be shown clearly was a shortening of the latent period after stimulation, before DNA
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synthesis reached a high level or gross morphological change occurred. Marshall, Roberts, Wanless & Young (1963), during continuous observation of the growth and division of human small lymphocytes in PHA cultures, noted that a blast cell from 1 small lymphocyte which divided after 67.5 h of culture gave rise to 2 daughter cells which divided synchronously after a further 23 h. In these cultures PHA was present throughout the experiment so that as soon as mitosis had occurred the progeny would be exposed to further stimulation. The shorter time for the second division would appear, from our observations, to be due to a shorter G1 period since DNA synthesis occurs sooner in restimulated cell populations. Similarly, using conditions in which PHA was present throughout the whole of the culture period Sasaki & Norman (1966) found that the transformation of human lymphocytes is essentially completed in 24-48 h and that after transformation the cells proliferate with a generation time of 22 h. At 72 h, 70-80 % of the cells in metaphase were found, on the basis of 3H-TdR chromatid labelling, to be in at least their second division. From the work of Buckton & Pike (1964) it appears that some of the metaphases present after only 48 h of culture are from lymphocytes at least 5 years old.

Two hypotheses would explain our results. First, that a given concentration of stimulant will cause activation of a proportion of the lymphocyte population, the rest of the lymphocytes being completely unaffected. This is, in essence, an all-or-none or 'trigger' hypothesis, a proportion only of the lymphocytes capable of undergoing activation being stimulated, depending on the dose of stimulant used. The alternative hypothesis is that each lymphocyte in the responsive population responds to a different degree, depending on the stimulant used and its concentration. The activation would then result in a transit through the cell cycle at a velocity related to the concentration of stimulant, lower doses giving slower but more prolonged DNA synthesis. This might be termed a 'push' hypothesis. In our experiments lymphocytes stimulated with a pulse of SF or PHA behaved as if pushed through the cell cycle. It is quite possible that a high level of stimulant may push a few highly susceptible cells through more than one cell cycle while other weakly responsive cells move only very slowly towards DNA synthesis and may not reach the synthetic period before the activity of the more responsive cells has been exhausted.

Rieke (1965) has examined the response of rat lymphocytes to PHA in vitro in relation to their life-span in vivo. He finds that 'young' lymphocytes respond more readily to PHA than 'old' lymphocytes. This finding, which agrees with the results in vitro reported in this paper, would imply that the lymphocytes of hyperimmunized animals might be stimulated more readily than those from untreated animals. Although we have not been able to demonstrate this in a limited number of experiments it still seems likely to be the case. We have been able to show that rabbit spleen contains a population of cells which responds to SF more quickly than peripheral lymphocytes from the same animal (Knight & Ling, unpublished observations). These early responders may again be young lymphocytes, possibly part of a splenic pool which is distinct from the pool of recirculating lymphocytes.

Holm & Perlmann (1965) have shown that lymphocytes activated with PHA have a heightened capacity to destroy allogeneic fibroblasts. On the same principle mixtures of
lymphocytes stimulated with PHA or SF might be expected to be mutually destructive. We found no evidence of this during the first 6 days after mixing but the fall-off in activity of the mixed stimulated cells after this time (see Fig. 8) might be interpreted in this way.

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Figs. 9-12. Autoradiographs of the progeny of stimulated lymphocytes. Figs. 9, 11 are human cells, Figs. 10, 12 are rabbit cells. x 3000.

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