Unstable Chromosome Changes in Tuberculin-Stimulated Leukocyte Cultures from Irradiated Patients. Evidence for Immunologically Committed, Long-Lived Lymphocytes in Human Blood

By Peter C. Nowell

Recently, the “small lymphocyte” has been reestablished as a useful member of human cellular society, and it has had a number of important immunologic and trephocytic functions assigned to it—often, however, on largely theoretical grounds. One such theory suggests that the peripheral blood of normal humans contains specifically “committed” small lymphocytes which may survive in the body for years without dividing, and yet retain the latent capacity to proliferate when reexposed to the sensitizing antigen. This concept implies that one’s “immunologic memory,” at least with respect to cellular immunity, resides in long-lived committed lymphocytes which, because they are not dividing, need not have undergone genetic alteration in the process of becoming sensitized.

Such a thesis has been proposed in part by many workers, and recently Fitzgerald has advanced the complete concept with a detailed review of the supporting data. In brief, these data include evidence that specific antigens can convert previously sensitized small lymphocytes to proliferating “blast” cells in human leukocyte cultures, and that small lymphocytes can survive for months or years in the body without dividing and yet can still proliferate in vitro when stimulated by phytohemagglutinin (PHA). The latter conclusion is based on the observation of “unstable” chromosome changes (dicentrics, acentrics, rings) in cultured lymphocytes from individuals irradiated months or years before. Such chromosome changes do not persist in cells for more than one or two cell divisions; so if these lymphocytes had been dividing in vivo during the postirradiation period, these unstable aberrations would have been eliminated from the cells.

To date, such studies on irradiated individuals have been done only with PHA-treated cultures. In the present paper, evidence is presented for the existence of long-lived human lymphocytes which can be caused to proliferate in vitro not only by PHA (which may act in a nonspecific manner), but also by a specific antigen (tuberculin).

Materials and Methods

Peripheral blood was obtained from patients who had received large doses of therapeutic radiation to major lymphopoietic areas 6 weeks to 3 years previously. The red

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...cells were removed by sedimentation or by immediate centrifugation (25 g, 10 min.) at room temperature. Five cultures were planted for each patient, using the technic of Moorhead et al.,\textsuperscript{5} with each culture bottle containing 10 million leukocytes in a mixture of 5 ml. TC-199, 2 ml. fetal calf serum, and 1 ml. autologous plasma. Two cultures received phytohemagglutinin (PHA) as a mitotic stimulant; two received Old Tuberculin (final dilution 1:1000); and one received neither. One culture containing PHA and one containing OT were terminated after 3 days, and the remaining 3 cultures were terminated after 5 days. In previous experiments, it had been determined that under these culture conditions, PHA-treated cultures usually showed their first and maximum wave of mitotic activity on day 3,\textsuperscript{6} with less activity on day 5; tuberculin-stimulated cultures (from sensitive individuals) usually showed their first and maximum activity on day 5, with rarely a few mitoses by day 3; and cultures without mitogenic stimulation usually showed no mitoses at all, with occasionally a very few appearing on day 5.

Colchicine was added 3 hours before termination, and chromosome preparations were made by the air-drying technic.\textsuperscript{5} Whenever possible, 100 good metaphases from each culture were counted and then scored for the presence or absence of three types of "unstable" chromosome aberrations: dicentrics, acentrics and rings. A cell was considered "aberrant" if it contained at least one of the three types of unstable abnormalities. In many instances, multiple aberrations were present. In this laboratory over the past 7 years, hundreds of cultures from nonirradiated individuals have been grown, and dicentrics and rings have not been observed; acentric fragments have been found in less than 1 per cent of cells from such cultures.

RESULTS

Thus far, eight irradiated patients have had sufficiently good mitotic activity in tuberculin-stimulated cultures to permit examination for unstable aberrations. The results are given in table 1, along with the patient's disease, the dose and site of irradiation, and the time between irradiation and culture. Blank spaces in the table indicate those instances in which fewer than 5 mitoses were seen in the entire culture. Note that except for two patients (numbers 167 and 81), this was the case with all 3-day tuberculin cultures. It was also true of all 5-day cultures which received neither PHA nor OT, and therefore this group has been omitted entirely from the table.

In the three patients studied 6 weeks to 6 months postirradiation (numbers 150, 169, and 167), unstable aberrations were present in the tuberculin-stimulated cultures as well as in the cultures treated with PHA. In patient number 150, the tuberculin-stimulated cultures grew poorly, but a few aberrant cells were observed. In patient number 167, the tuberculin-treated cultures grew unusually well, with mitotic activity noted at 3 days, while the cultures stimulated with PHA were surprisingly poor. This patient was restudied two weeks later and the same result was obtained. Unstable changes were observed in OT-treated cultures on both occasions (fig. 1), and were also present in the 5-day tuberculin-stimulated culture of patient number 169.

In contrast to these findings, unstable aberrations were extremely rare in the tuberculin cultures of the 5 patients studied more than a year after irradiation. Although these changes were still common in their PHA-treated cultures, a total of only 3 unstable aberrations were found in all of the tuberculin cultures: two ring chromosomes (fig. 2) and a small acentric fragment. Interestingly, however, in the tuberculin culture of one of these five patients, number 134,
Table 1.—Chromosome Aberrations in PHA- or OT-Stimulated Leukocyte Cultures from Irradiated Humans

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>X-Ray Dose</th>
<th>Site</th>
<th>Time Post-Irradiation</th>
<th>Unstable Aberrations (Aberrant Cells/Total Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PHA (5 days) PHA (5 days) OT (5 days) OT (5 days)</td>
</tr>
<tr>
<td>150</td>
<td>Lymphosarcoma</td>
<td>1000 r</td>
<td>Upper abdomen</td>
<td>1½ mo.</td>
<td>31/100 3/21 — 4/31</td>
</tr>
<tr>
<td>169</td>
<td>Carcinomas, esophagus</td>
<td>3900 r</td>
<td>Neck and thorax</td>
<td>6 mo.</td>
<td>— 15/100 — 7/100</td>
</tr>
<tr>
<td>167</td>
<td>Gastric hemorrhage</td>
<td>1670 r</td>
<td>Upper abdomen</td>
<td>6 mo., 6½ mo.</td>
<td>7/34 3/42 8/53 4/100</td>
</tr>
<tr>
<td>158</td>
<td>Carcinoma, mediastinum</td>
<td>2000 r, 3000 r</td>
<td>Neck, Mediastinum</td>
<td>15 mo., 20 mo.</td>
<td>4/100 0/100 0/100</td>
</tr>
<tr>
<td>175</td>
<td>Lymphosarcoma</td>
<td>1840 r, 1560 r</td>
<td>Lower abdomen, Spleen</td>
<td>17 mo., 72 mo.</td>
<td>31/100 5/100 1/100</td>
</tr>
<tr>
<td>165</td>
<td>Chr. lymphocytic leukemia (remission)</td>
<td>2000 r</td>
<td>Spleen</td>
<td>24 mo.</td>
<td>14/100 1/34 — 0/100</td>
</tr>
<tr>
<td>134</td>
<td>Lymphosarcoma</td>
<td>2000 r</td>
<td>Spleen</td>
<td>24 mo.</td>
<td>41/100 18/100 — 0/90*</td>
</tr>
<tr>
<td>81</td>
<td>Polycythemia vera (remission)</td>
<td>1200 r</td>
<td>Spleen</td>
<td>38 mo.</td>
<td>9/100 2/100 1/23 1/100</td>
</tr>
</tbody>
</table>

*Abnormal stemline (46 chromosomes).

an aberrant stemline with a stable chromosome change was present. Forty-one of the 90 metaphases counted in this culture contained only 45 chromosomes, and in 6 of 7 karyotype analyses a chromosome of the large X-6-12 group (C-group) was missing. This was the only culture in this or any other patient in which an abnormal stemline could be definitely identified, although other stable aberrations, generally the products of deletions or translocations, were seen in occasional cells in all cultures.

Table 1 also indicates that in those individuals in which the same mitogenic stimulant (PHA or OT) produced mitoses on both days 3 and 5, the frequency of aberrant cells was always lower in the later culture, when many of the cells were apparently in their second division in vitro. Similar observations have been reported by Buckton and Pike⁷ and constitute further evidence that unstable aberrations do in fact tend to be progressively eliminated with each cell division.

**Discussion**

The present findings generally support the hypothesis described in the Introduction. There appear to be, in the circulation, immunologically committed lymphocytes which can survive in the body for at least 6 months without dividing and which can still respond by proliferation to specific immunologic challenge (tuberculin). The extreme rarity of these cells beyond 6 months in this study may only be an indication of the frequency with which the average individual is exposed to tubercle bacilli. Any exposure following irradiation
Fig. 1.—Dicentric chromosome (arrow) and small acentric fragments in a metaphase from a 3-day tuberculin-stimulated leukocyte culture. Blood sample from patient #167, 6 months after upper abdominal x-irradiation for gastric hemorrhage.

Fig. 2.—Large ring chromosome in metaphase from a 5-day tuberculin-stimulated culture. Blood sample from patient #81 (female), 3 years after splenic irradiation for thrombocytopenia associated with polycythemia vera.

should cause the cells committed to this antigen to proliferate, with loss of unstable chromosome changes, and apparently the five patients reported here who were examined more than 6 months after irradiation had already undergone such exposure.

On the other hand, unstable changes were still present in the PHA-treated cultures of these five patients. Since PHA apparently nonspecifically stimulates all committed lymphocytes to proliferate,\textsuperscript{3,4} the cells dividing in these cultures would include many committed to antigens much less common in the environment than tuberculin. Such cells might well persist in the body for years without encountering antigenic stimulation, and thus would still show unstable aberrations when caused to proliferate by PHA.

In this connection, the findings in patient number 134, 2 years following irradiation, are of interest. Unstable aberrations were present in the PHA-treated cultures, while in the tuberculin culture there was only a stable abnormality, a stemline with 45 chromosomes. Exposure to tubercle bacilli at some time during the postirradiation period could well have caused proliferation of tuberculin-committed lymphocytes with elimination of their unstable aberrations, but with retention of the stable abnormality in one clone of newly committed
small lymphocytes. At the same time, the cells with unstable changes in the PHA-treated cultures would represent lymphocytes committed to other antigens and which had not encountered these antigens during the postirradiation period.

Obviously, before these concepts can be fully substantiated, more human studies are needed, employing a wide variety of antigens, including ones less common than tuberculin, and with a greater range of postirradiation times. In addition, similar studies in laboratory animals would be desirable since sensitization and irradiation could be precisely controlled, and also the possibility of temporary sequestration of the committed lymphocytes in various organs could be explored. Gowans et al. and Porter and Cooper have presented in vivo evidence of conversion of injected small lymphocytes into proliferating, immunologically competent “hemocytoblasts” in homologous hosts. However, these studies provide no information on the life span of the injected cells. Personal attempts to obtain in vitro data (similar to those reported here for humans) with rats, rabbits and guinea pigs have thus far been unsuccessful.

Finally, it may be well to attempt to anticipate certain criticisms of the present study. Since therapeutic irradiation for benign conditions is no longer common, the available patients generally were suffering from malignant disease, often of hematopoietic tissues. Fortunately, the patient providing the best evidence of tuberculin-stimulation of long-lived cells, patient number 167, was irradiated in order to control gastric hemorrhage and had no hematopoietic disorder. As more data are accumulated, however, it may be necessary to consider the patient’s disease in evaluating the results.

Perhaps even more important is the question of the validity of using unstable chromosome changes as a marker. Certainly, it now seems well documented that unstable chromosome changes are rapidly eliminated from dividing cell populations and are only observed in cells which have not been proliferating. Earlier data in lower organisms have recently been supplemented by similar findings in a number of mammalian species, including man. However, one cannot state with assurance that, either in vivo or in vitro, a radiation-damaged cell functions in the same way as its undamaged counterpart. There is as yet no evidence that the radiation-damaged cell which we are using as a marker is not a valid representative of the lymphocyte population which we are attempting to study, but until some other type of marker permits an independent check on the conclusions presented, this possibility cannot be excluded.

**Summary**

Unstable chromosome aberrations (acentrics, dicentrics, rings) have been observed in tuberculin-stimulated leukocyte cultures from three patients up to six months after therapeutic irradiation. This is considered evidence that human blood contains immunologically committed, long-lived, nondividing small lymphocytes which retain the latent capacity to proliferate when re-exposed to the sensitizing antigen. “Immunologic memory” may reside in such cells, which, because they are not continually dividing, need not have undergone genetic alteration in becoming immunologically committed.
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SUMMARIO IN INTERLINGUA

Instabile alterationes chromosomatic (acentricitate, dicentricitate, annularitate) esseva observate in culturas de leucocytos pre-stimulate con tuberculina e obtenite ab tres patientes subjicite a irradiation therapeutic usque a sex menses previemente. Iste constatation es considerate como evidentia in favor del thes que le sanguine human contine immunologicamente "dedicate," longeve, e nonmitotisante micre lymphocytos le quales retine le latente capacitate de proliferar quando illos es re-exponite al antigeno sensibilisante. "Memoria immunologic" reside possibilemente in tal cellulas, le quales—proque illos non es continuamente dividite—non debe haber experientiate un alteration genetic in devenir immunologicamente "dedicate."

ACKNOWLEDGMENTS

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ADDENDUM

Patient number 167, originally studied 6 and 6½ months after irradiation, was restudied at 11 months. Unstable aberrations had completely disappeared from the OT cultures, although their frequency was only slightly reduced in 3-day PHA cultures (10 per cent vs. 13 per cent). These findings fit with the other data suggesting that tuberculin-sensitized cells may be frequently exposed to antigen in vivo resulting in early elimination of unstable changes. However, the possibility that the lymphocytes stimulated by tuberculin and those responding to PHA represent 2 entirely separate populations has not been excluded.

REFERENCES


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