The Development of Large Immature Mononuclear Cells in Mixed Leukocyte Cultures

By Barbara Bain, Magdalene R. Vas and Louis Lowenstein

When human peripheral blood leukocytes are cultured in vitro in the presence of phytohemagglutinin, some of the cells transform into immature forms that are capable of mitotic division. An incidental observation by Schrek and Donnelly indicated that, under certain conditions, a similar reaction might occur without phytohemagglutinin. These authors were studying the morphology and motility of lymphocytes from 20 leukemic and non-leukemic patients, using the slide-chamber method and time-lapse cinematography. In one of their cultures, after 5 days' incubation, they observed "a small number of large cells with large clear nuclei and prominent nucleoli." A few of the cells were in mitosis. The authors noted that the culture "proved to be a mixture of bloods from 2 patients with hemochromatosis," but did not elaborate on this observation. Since this one culture differed from the others only in that it was a mixture of leukocytes obtained from two subjects, it seemed desirable to us to determine whether the large cells would appear consistently when leukocytes from two normal individuals were mixed together and cultured in the absence of phytohemagglutinin.

Materials

The subjects were normal volunteers, except for one who had rheumatoid arthritis and a positive L.E. test, and another who had a virus infection with atypical lymphocytes in the peripheral blood. With the exception of the twin pairs, all were unrelated to one another.

Methods

The technics were adapted, with modifications, from standard methods that have been used by many workers. The leukocytes and other blood elements were subjected to as few manipulations as possible.

a. Culture Methods

Venous blood was drawn into a heparinized syringe and allowed to sediment for 1 hour in a 15 ml. centrifuge tube. Maintaining the tube at 37 C. was found to give the best yield of leukocytes. The blood was then centrifuged at 25 g for 5-10 minutes, and the supernatant plasma, containing leukocytes, platelets, and a few erythrocytes, was drawn off. The supernatant was diluted with TC-199 medium* (containing penicillin 100 units/ml. and streptomycin 100 μg./ml.) so that the leukocyte count was between 800

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and 2000/mm³. The plasma concentration varied from 10 to 20 per cent, and was adjusted occasionally by adding cell-free plasma to the cell suspension. For each culture, 4 ml of cell suspension was placed in a 17 x 100 mm. disposable plastic culture tube and kept at 37 C. The leukocytes were allowed to settle to the bottom of the tube. In the basic experiments, cultures consisted of a 1:1 mixture of cells from the two subjects, with controls being cultures of cells from each subject alone.

b. Effect of Homologous Erythrocytes, Plasma and Platelets

Two subjects were used in each experiment, and the culture methods were basically the same as those described in the previous section. Erythrocytes, plasma or platelets from the first subject were added to leukocytes from the second subject, and vice versa. Leukocyte mixtures and unmixed control cultures were also prepared from each pair of subjects.

The effect of foreign erythrocytes on a single set of leukocytes was determined by taking a sample from the bottom of the tube containing blood from one subject, after the initial sedimentation and slow centrifugation. A small portion of this sample was added to a suspension of leukocytes obtained from the other subject. This resulted in approximately 5000 foreign erythrocytes/mm³, or about 10 times the number of erythrocytes usually present. In these cultures there were no more than 2 foreign leukocytes/mm³, which was insignificant when compared with approximately 1000 autologous leukocytes/mm³.

To determine the influence of leukocytes and plasma separately, the supernatant was obtained in the usual way. It was then centrifuged for 5 to 10 minutes at 400 g. The cell button was washed twice with medium and the cells were resuspended in a small amount of medium. The plasma was centrifuged again to remove any remaining cells. Cell suspensions and plasmas were diluted with medium so that the cultures contained the same leukocyte and plasma concentrations as described in the section on culture methods. Leukocytes from one subject were incubated with plasma obtained from the other, and, as a control, the same leukocytes were suspended in autologous plasma.

In the experiments designed to test the effect of platelets, blood from each subject was divided into two equal aliquots. One aliquot (No. 1) was allowed to sediment to obtain a leukocyte suspension. The other (No. 2) was immediately centrifuged for 5 minutes at 400 g. The supernatant obtained from this second aliquot contained no leukocytes that could be detected by counting. Only a very occasional leukocyte was seen in the stained smears, but large numbers of platelets were present in the smears. Platelet counts from both supernatant samples were approximately equal to one another. Supernatant No. 1 from one subject was then mixed with an equal amount of supernatant No. 2 from the other subject.

c. Staining Methods and H³-thymidine Autoradiographs

Smears were made from the cultures after 5 days' incubation, and stained with Jenner-Giemsa. A few smears were stained with Pyronin Y and Methyl green. Also after 5 days, H³-thymidine autoradiographs were prepared. The H³-thymidine (s.a. 5.9 or 17.8 mc./mg.) was added to the cultures to give an activity of 1 mc./ml. After 1 hour at 37 C., the cultures were centrifuged, the fluid discarded and the cells smeared on slides and dried. The slides were fixed for 3 minutes in absolute methanol, washed, dried and coated with Kodak NTB-2 liquid nuclear track emulsion. After 2 weeks' exposure at 5 C., they were developed with Kodak Dektol developer. The slides were stained with Giemsa or Jenner-Giemsa; 1000 leukocytes were counted under the microscope and the number of labeled cells was noted.

RESULTS

a. Basic Experiments with Leukocyte Mixtures and Controls

1. Stained smear morphology: After 5 days' incubation, smears made from the mixed cultures from non-related subjects and stained with Jenner-Giemsa
Fig. 1.—Large basophilic cell from mixed leukocyte culture. Also shown are a small lymphocyte, and a cell which is intermediate in size and chromatin pattern. Five days’ incubation. Jenner-Giemsa stain.

Fig. 2.—Mitosis in mixed leukocyte culture. Five days’ incubation. Jenner-Giemsa stain.

Fig. 3.—Large cell from mixed leukocyte culture. Five days’ incubation. Methyl green-pyronin Y stain.

Fig. 4.—H3-thymidine autoradiograph: labeled large cell in mixed leukocyte culture. Five days’ incubation. Jenner-Giemsa stain.

showed a considerable number of very large cells with basophilic cytoplasm, a fine, evenly distributed chromatin pattern, and prominent and abundant nucleolar material (fig. 1). Some of these cells were in mitosis (fig. 2). In smears stained with Methyl green and Pyronin Y, their cytoplasm was intensely pyroninophilic (fig. 3). Some cells were seen whose size and chromatin pattern suggested that they might be intermediate between lymphocytes and the large basophilic cells (fig. 1). Most of the neutrophils had disappeared after 5 days, and the few that were present were extremely degenerated. Small lymphocytes and eosinophils, however, were relatively unchanged from their appearance in fresh blood. Some mononuclear cells with eccentric nuclei also were present, with cytoplasm containing granules of a variety of shapes and sizes. These cells ingested India ink particles which had been added to the cultures 2 hours before the smears were made.
In contrast to the mixtures, very few of the large basophilic cells appeared in the control cultures. Otherwise, the controls were similar to the mixtures, containing mainly small lymphocytes and phagocytic cells, with a few eosinophils and degenerating neutrophils.

2. H\(^3\)-thymidine autoradiographs: A general impression of the reaction in the mixed cultures could be obtained from the examination of stained smears, but a more quantitative approach was possible with the use of H\(^3\)-thymidine autoradiographs. These were all prepared after 5 days' incubation. About half of the large basophilic cells were labeled as illustrated in figure 4. No other cells incorporated H\(^3\)-thymidine, except for an occasional cell of the previously described intermediate type.

The increase in the number of labeled cells in the leukocyte mixtures was striking as compared with the unmixed controls (fig. 5). There was considerable variation in both mixtures and controls and some overlapping between the two groups (fig. 6-I, II), but in each individual experiment the mixture showed more labeling than either of the two single sets of leukocytes.

Total leukocyte counts were done at the end of the culture period and no consistent differences were found between mixtures and controls. Blood grouping was performed on all subjects, and similarities or differences between ABO blood groups did not influence the results; most of the subjects were Rh positive.

In these basic experiments (figs. 5; 6-I, II), and in the results to be described (figs. 6-III, IV, V; 7), there was good correlation between the H\(^3\)-thymidine labeling results and the appearance of the stained smears: an increased number of labeled cells was found only if the smears showed an increased number of large basophilic cells in comparison with the controls.
Fig. 6.—Leukocyte cultures from non-related subjects. Each point represents one culture; horizontal lines are mean values. Five days’ incubation. H3-thymidine autoradiographs. I. Unmixed controls—one donor. II. Leukocyte mixtures—two donors. III. One donor’s leukocytes; foreign erythrocytes added. IV. One donor’s leukocytes; foreign plasma added. V. One donor’s leukocytes; foreign platelets added.

b. Effect of Various Blood Elements

When plasma, erythrocytes or platelets from one subject were added to the leukocytes from another subject, there was no reaction (fig. 6-III, IV, V). Mixtures of leukocytes from the same pairs of subjects showed an increase in H3-thymidine labeling.

c. Preliminary Studies with Twins

These results are shown in figure 7.

There was no reaction between the leukocytes of the three pairs of monozygotic twins that were studied. These twins previously had been investigated extensively. Blood grouping, fingerprint analysis, and similarity with respect to a number of other physical traits had indicated that each pair was identical. One pair (No. 3) had been studied with particular care. Five years previous to this study, one twin had received a kidney transplanted from the other, and before the operation they had successfully undergone reciprocal skin grafting. Both twins are now healthy and the transplanted kidney is functioning well. The case has been reported by Dossetor et al.3

The results with the dizygotic twins were variable. Two pairs (No. 4 and 5) showed reactions comparable with those seen between the leukocytes of unrelated subjects, whereas the other two pairs (No. 6 and 7) showed no reaction. The twins who were studied in experiment No. 6 were the only ones who were not proven to be dizygotic on the basis of sex difference or blood grouping, but they definitely were not identical in appearance.

Discussion

The basophilic mononuclear cells in the mixed leukocyte reaction resemble morphologically those cells which develop in vivo in lymph nodes and spleen in response to injection of soluble antigens4,5 or following skin homografting.6
The origins and transformations of such “basophilic lymphoblastoid cells” have been reviewed by Berman. The development of similar cells in vitro in leukocyte mixtures may be stimulated by antigenic differences between the leukocytes from two individuals. Medawar investigated the distribution of transplantation antigens in the various blood elements. He obtained accelerated rejection of skin homografts in mice and rabbits which had previously received intradermal injections of leukocytes from the skin donor, whereas previous injections of the donor’s erythrocytes, plasma or platelets had no such effect. The ineffectiveness of foreign plasma, erythrocytes or platelets in stimulating the leukocyte response in our experiments suggests that similar factors may be concerned in our vitro system and in the in vivo homotransplantation reaction. Ebbe et al. have shown that antigens which affect the survival of skin homografts may be present in rabbit platelets. However, their results indicate that these antigens may be weak or unstable, so that they might not have any demonstrable effect in an in vitro system.

Our results with pairs of twins do not permit any definite conclusions at present, but a comparison of these results with the finding of a consistent reaction between leukocytes from non-related subjects suggests that genetic factors may be involved. The possibility of the development of immunologic tolerance between dizygotic twins must also be considered. In dizygotic cattle twins, prenatal exchange of blood cells is commonly found and results in blood group chimerism and tolerance towards reciprocal skin grafts. The same situation is apparently rare in human twins, but it has been reported. By comparing the reaction in leukocyte mixtures from dizygotic twins, pairs of siblings and pairs of non-related subjects, it may be possible to estimate the relative effects of genetic similarity and induced tolerance.
Although not conclusively proven, several lines of evidence indicate that the large basophilic cells in the leukocyte mixtures are derived from small lymphocytes. Examination of the stained smears from the mixtures showed cells intermediate between these two forms. When small lymphocytes from rat thoracic ducts were injected into lethally irradiated mice, Gowans et al. found that the rat lymphocytes were transformed into large, dividing pyroninophilic cells in the white pulp of the mouse spleen. A similar experiment was performed by Porter and Cooper using two strains of rats. The addition of phytohemagglutinin to cultures of peripheral blood leukocytes results in the appearance of blast-like cells, many of which are in mitosis, and which arise by the transformation of a relatively large cell population, probably the small lymphocytes.

Recent findings have suggested that the action of phytohemagglutinin may be due to its antigenic nature, and tuberculin and other vaccines have been shown to have the same effect on leukocytes in vitro. The transformed cells in these cultures contain gamma globulin. Although it is less intense and involves changes in a smaller proportion of the lymphocytes, the mixed leukocyte reaction is qualitatively similar to the effect of phytohemagglutinin. The antigenic stimulus in this case would be the foreign leukocytes.

There is some evidence that the response to an antigen may require its ingestion and modification by phagocytic cells. In our leukocyte mixtures, as well as in the unmixed controls, cells were present whose phagocytic abilities were demonstrated by their capacity to ingest India ink particles. Favier et al. have also found phagocytic mononuclear cells in cultures of leukocytes from single individuals. The same type of cell has been described in the past by a number of authors, and more recently by Rabinowitz and Schrek, who believed that these cells were of monocytic origin.

The reaction that is observed in mixed leukocyte cultures may form a basis for a quantitative approach to the problem of homograft immunity in man. The response of different species to homografts is variable, so the results of animal experiments cannot always be applied to humans. Therefore, there would be obvious advantages in using human leukocytes, in an in vitro system that allows close control of the experimental environment. Such a technic might also prove to be valuable in predicting the compatibility between the prospective donor and the recipient of a homograft. The test material, peripheral blood, is easily available, and the procedure would not involve any danger of sensitization of either donor or recipient.

**Summary**

1. When leukocytes from two normal, unrelated subjects are mixed together and cultured, some of them transform into large basophilic cells that can synthesize DNA and undergo mitosis.
2. The stimulus for this change is the presence of two sets of leukocytes. Foreign erythrocytes, plasma or platelets have no effect.
3. Studies with mixtures of leukocytes from pairs of identical and non-identical twins indicate that the reaction may be related to genetic differences between the two subjects.
4. It is suggested that the reaction in leukocyte mixtures may be related to homograft immunity.

**Summario in Interlingua**

1. Quando leucocytos ab duo normal non-consanguinee subjectos es miscite e culturate insimul, certes inter illos se transforma in grande cellulas basophilic que es capace a synthetisar acido disoxyribonucleic e a experientiar mitosis.

2. Le stimulo pro isto alteration es le presentia de duo typos de leucocytos. Erythrocytos o plasma o plachettas hetero-originari es sin effecto.

3. Studios con mixturas de leucocytos ab pares de geminos identic e non-identic indica que le reaction es possibilemente relationate con differentias genetic inter le duo subjectos.

4. Es suggestionate que le reaction in mixturas de leucocytos es forsang relationate con le phenomeno del immunitate de homograffage.

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