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12 May 1966

One-Way Stimulation in Mixed Leukocyte Cultures

We have developed an improved method for the mixed leukocyte culture test. Control values, as determined by rates of incorporation of thymidine, are very low, allowing evaluation of low levels of stimulation in homologous cell mixtures. One-way stimulation is assayed by treating the cells of one individual with mitomycin C; treated cells cannot respond (incorporate thymidine) but can still stimulate homologous untreated cells to do so.

Determination of histocompatibility in man by use in vitro of mixed leukocyte cultures (MLC) is receiving attention. The original observation was that mixtures of leukocytes from two genetically unrelated individuals resulted in mutual stimulation, evidenced by enlargement and mitosis of lymphocytes in culture. The method seemed to have potential as a measure of histocompatibility because (i) mixtures of cells from identical twins did not result in stimulation (*1, 2*), (ii) mixtures of cells from related individuals resulted in less stimulation, on the average, then mixtures of cells from unrelated individuals (*3*), and (iii) the histocompatibility status of one set of three individuals, based on MLC determinations, correlated with their compatibilities determined by the third-man test (*2*). Other reported data (*4, 5*) suggest that the test does provide some measure of histocompatibility in man.

Some undesirable features in the method described by Bach and Hirschhorn (*2*) have become apparent: High control values in cultures containing cells from one individual make it difficult to evaluate the significance of low levels of stimulation in mixed cultures; although the values are reduced by use of human serums instead of fetal calf serum (*6*) in the medium, control values still represent up to 20 percent of values obtained in mixed cultures of incompatible cells. Furthermore, cells from both individuals under test are able to respond to each other; it is obviously desirable to measure one-way stimulation (*7*)—wherein the contribution of each individual's cells to the total response can be measured independently.

We now describe an improved method for MLC testing in which (i) control values, as determined by rates of thymidine incorporation, are very low —in many instances zero; and (ii) one-way stimulation is assayed by treating the cells of one of the test subjects with mitomycin C to prevent DNA replication (*8*). Such treatment prevents the cells from acting as "responding cells" (responding to homologous tissue by enlarging and incorporating thymidine) without preventing them from acting as "stimulating cells" (stimulating homologous cells to respond) (*9*).

Freshly drawn heparinized blood is allowed to sediment in 25- by 150-mm glass tubes for 2 hours at 37°C; subsequent procedures preceding culture are at room temperature. The plasma is centrifuged for 10 minutes at 150*g* to obtain leukocytes; the supernatant is recentrifuged for 10 minutes at 1000*g* to provide cell-free plasma. Cells to be tested as "responding cells" are suspended in Eagle minimal essential medium (*10*) modified for suspension culture (MEM-S, Grand Island Biological) and supplemented to contain glutamine, 2 m*M*; penicillin, 100 units per milliliter; streptomycin, 100 μg/ml; and 20 percent autologous cell-free plasma.

Cells to be used as stimulating cells are suspended at a concentration of 2 to 10 $\times$ 10^6 leukocytes per milliliter in MEM-S containing 10 percent autologous plasma, incubated for 20 minutes at 37°C with mitomycin C at 25 μg/ml, twice washed in Hanks buffered salt solution containing 10 percent plasma from the donor of the responding cells, and then suspended in MEM-S containing 20 percent plasma from the donor of the responding cells. Stimulating cells are mixed with responding cells to give final cell concentrations of, respectively, 7 to 10 $\times$ 10^5 (leukocytes) and 5 $\times$ 10^5 (lymphocytes) per milliliter. The mixture is immediately distributed in 2.5-ml volumes in 16- by 100-mm glass tubes with metal closures, and incubated at 37°C in a humidified 4 percent CO_2 atmosphere for 7 days.

After approximately 160 hours of incubation, 2 μc of tritiated thymidine (specific activity, 1.9 c/mmole; Schwarz BioResearch) is added to each culture. Replicate cultures are harvested 1.5, 3.5, and 5.5 hours later by addition of 1000-fold excess of nonradioactive thymidine in 0.1 ml of normal saline, immersion of the culture tubes in ice, centrifugation for 10 minutes at 1000*g*, and freezing of the cell sediment at −20°C. To determine incorporation of thymidine the cultures are thawed, 5 ml of cold 5-percent trichloroacetic acid is added to each, and the tube contents are mixed on a Vortex mixer. Ten minutes later the precipitate is recovered by centrifugation, dissolved in 1 ml of 0.1*N* NaOH, and precipitated again with 4.5 ml of 6.7-percent trichloroacetic acid. The acid-precipitation procedure is repeated twice again. The final precipitate is dissolved in 0.1 ml of tetraethylammonium hydroxide, mixed with scintillation fluid (*11*), and counted; the results are expressed as counts per minute (cpm) in each sample. Reproducibility of the assay was determined with 150 mixtures, each mixture being cultured in triplicate; 82 percent of all triplicates showed counts within ± 12 percent of their mean; 97 percent, within ± 18 percent. Six of the 450 samples were disregarded because they deviated from the mean of the triplicates by more than 60 percent (always on the low side); they probably reflected some unusual technical accident.

Responding cells (A), incubated with autologous mitomycin C–treated stimulating cells (A_m), serve as a control for homologous mixed cultures (for example, AB_m, AC_m, and such); thus is enabled comparison of the reactions of cells of one individual (a potential recipient) with reactions of the homologous cells of a series of other individuals (potential donors).

Fig. 1*A* shows the results of culturing together the responding cells (F) of one individual with his own stimulating cells (F_m) and with those from another individual (G_m). Label was added after 160 hours and triplicate cultures were harvested at the times shown. Four slopes representing rates of incorporation were obtained. While the rate in

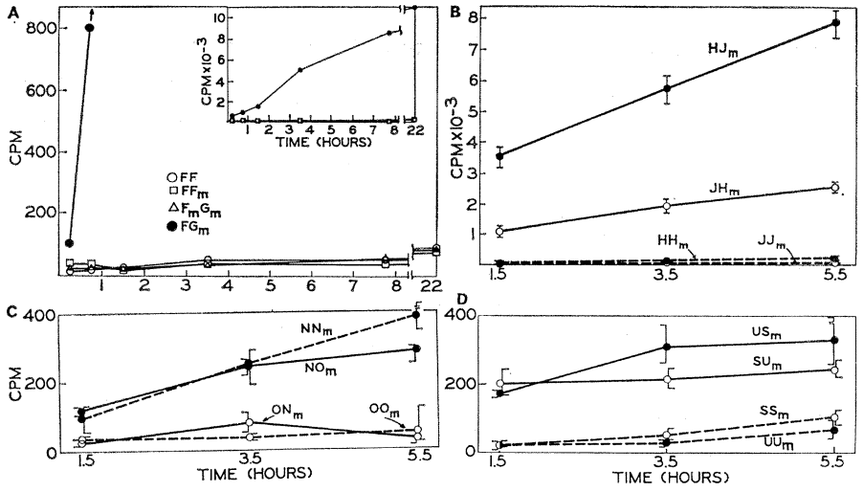

Fig. 1. Incorporation of H^3-thymidine into acid-precipitable material in different mixed leukocyte cultures; the H^3-thymidine was added to the cultures after they had been incubated for 160 hours. Replicate cultures were harvested at the times indicated after the addition of label. *A*, A long-term experiment, using cells of two unrelated individuals; FG_m and FF_m represent the response of untreated cells (F) of one individual to the treated cells (G_m) from the second individual and to his own treated cells (F_m); FF and F_mG_m and other mixtures; the inset is on a different scale to permit all the data to be plotted. *B*, Rate studies with cells, of two unrelated individuals, tested in reciprocal (JH_m and HJ_m) mixed cultures with their respective controls (JJ_m and HH_m). *C*, Reciprocal rate studies, using two siblings whose cells did not stimulate either by rate criteria or by single-point measurements. *D*, Reciprocal rate studies, using two siblings whose cells did not stimulate by rate criteria, but in which single-point comparisons could be otherwise interpreted.

the culture FG_m is markedly positive, the rates of incorporation into the cultures FF, FF_m, and F_mG_m are negligible. The virtual identity of the last three rates—assayed far beyond the usual time—indicates that cells treated with mitomycin C are rendered incapable of acting as responding cells in MLC, while they retain ability to stimulate (FG_m); and that addition of autologous treated cells (F_m) to cells able to respond (F) does not cause them to respond (FF_m).

The results of use of the standard method with cells of two unrelated individuals, H and J, appear in Fig. 1*B*. Incorporation of thymidine is close to a linear function of time from 1.5 to 5.5 hours. In both HJ_m and JH_m (reciprocal one-way cultures), positive stimulation is significant. Reciprocal one-way studies with two sets of siblings whose cells did not stimulate each other are demonstrated in Fig. 1, *C* and *D*. Figure 1*C* shows results with cells from N and O; the very close identity of the controls (OO_m and NN_m) with their respective homologous mixtures (ON_m and NO_m is striking, despite the slightly positive slope of NN_m.

Figure 1*D* shows the results with cells from S and U; these cultures also show no stimulation as judged by comparison of rates of incorporation (cpm incorporated per hour of labeling) of the control and mixed cultures. On the other hand, simple comparison of values of thymidine incorporation in the US_m and UU_m or SU_m and SS_m cultures at any single time might lead to a different conclusion.

The curves in Fig. 2 represent five different homologous mixtures. Responding cells at a fixed concentration show a variable response as a function of the number of stimulating cells. However, the pattern of response to increasing concentrations of stimulating cells is variable in the different mixtures, peak stimulation occurring at different numbers of stimulating cells. The variation shows no clear correlation with either the genetic relation of the two individuals under test or the cellular composition of the stimulating cells; thus is made difficult comparison of degrees of stimulation. We have been most interested in more clearly defining nonstimulating mixtures, which may well be the most interesting for genetic studies and histocompatibility testing.

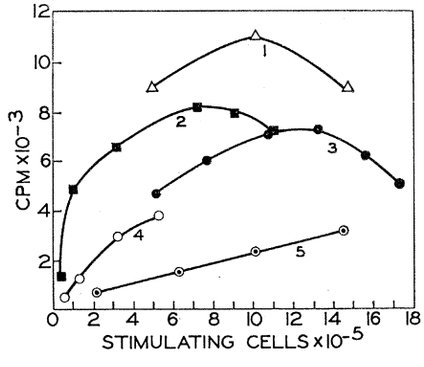

Fig. 2. Representative curves showing the response of a fixed number of responding cells to variation in the number of stimulating cells; each curve represents a mixture of cells from a different set of two individuals.

Validity of the method we describe is based on one-way studies of 91 different mixtures, 23 of which were studied by rates and 68 by single-point assay after 5.5 hours of labeling. Included in the 23 rate studies were eight mixtures that resulted in no stimulation; the eight were repeated on at least two other occasions; repeated rate studies again showed zero stimulation, and results of one-way single-point measurements and two-way stimulation were consistent with no stimulation. Eleven of the rate studies showing stimulation were repeated with the use of rates and four more were repeated with the use of single-point assays; again all mixtures were clearly positive. Although no attempt was made to repeat all 68 stimulating one-way single-point studies, 27 were done at least twice and remained positive. In all 46 cases tested by both one-way and two-way stimulation, results by the two methods have agreed. Of 27 stimulating reciprocal one-way studies (54 different matches), none showed positive stimulation in one direction and zero stimulation in the other.

Two experiments done on successive days early in this investigation gave apparently contradictory results on repetition—change from zero to positive stimulation. Any of many conceivable technical errors may have been responsible, and to detect such errors we have adopted a minimum set of criteria for accepting nonstimulation: If a mixture AB_m shows no stimulation, this result will only be accepted if, in the same experiment, the test cells (A) have been shown to be capable of reacting as responding cells; and if the treated cells (B_m) have been shown to be capable of acting as stimulating cells. Zero stimulation can thus be defined if the above criteria are met and if the rate of incorporation in a mixed culture (AB_m) does not exceed the rate in the control culture (AA_m) for that mixture.

Eight instances of nonstimulation have been observed, all in matches between siblings. Whereas 25 percent

(8/32) of all sibling mixtures studied by one-way testing have not stimulated, no case of nonstimulation has been observed in one-way testing of 52 mixtures of cells from unrelated individuals. These data are in agreement with our earlier data obtained by use of two-way stimulation and with the two-way data of Bain *et al.* (*3*). In one-way studies one would expect that 25 percent or more of sibling mixtures would not stimulate if the genotype at only one locus controlled the presence of factor(s) responsible for stimulation, regardless of the number of different alleles distributed in the general population, since a maximum of four alleles can be represented in a two-generation family at any one locus. If one uses the definition of zero stimulation expressed in this report, the simplest hypothesis—but not the only one—compatible with the present data is that stimulation in the MLC test results from difference between the stimulating cells and the responding cells controlled by the genotype at a single locus (*5*).

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Hepatic Synthesis of Alpha$_2$ (Acute Phase)-Globulin of Rat Plasma

Abstract. *Synthesis of plasma alpha$_2$ (acute phase)-globulin was demonstrated in isolated perfused rat liver obtained from animals showing acute inflammatory reaction to injury. These findings indicate that the liver is a source of the globulin and that appearance of this protein in the serum results from* de novo *synthesis by the liver rather than from release of performed and stored globulin.*

An alpha globulin, referred to as "slow" α_2-globulin because it migrates with the mobility of an α_2-globulin on paper electrophoresis but more slowly than β-globulin on vertical starch-gel electrophoresis, has been detected in the serum of animals under widely varying diverse pathological and experimental conditions, including acute inflammatory reaction to injury (*1*). This serum protein has also been designated α_2 (acute phase)-globulin (*2*). Observations that this singular α-globulin cannot be detected in the serum of normal adult animals have stimulated curiosity as to its origin. Because isolated perfused rat liver has been successfully employed to demonstrate hepatic synthesis of serum orosomucoid (*3*) and haptoglobulin (*4*), it appeared that the same system might serve to determine whether the liver is a site of synthesis of this acute-phase globulin. We have demonstrated synthesis of α_2 (acute phase)-globulin in plasma with isolated perfused rat liver that had been obtained from animals showing acute inflammatory reaction to injury.

The rat-liver perfusion system has been detailed (*3*). The donors of liver and blood were (fed) males of the Sprague-Dawley strain weighing 350 to 400 g. Acute inflammation was produced by daily subcutaneous injection of 1.0 ml of commercial-grade steam-distilled wood turpentine for 3 days before livers and whole blood were taken from the injected animals. In control experiments comparable amounts of "inflammatory" blood and radioactivity were circulated without the presence of the liver. Synthesis by normal livers was studied with liver and whole blood obtained from normal rats. At zero time, 100 μc of DL-(4,5-H^3)leucine (5.45 c/mmole) (*5*) was added to the blood perfusing the livers, and the plasma was sampled at intervals during 3 hours of perfusion.

To obtain specific antiserum, rabbits were immunized at 10 to 14 day intervals with an acute-phase α_2-globulin fraction of rat serum, obtained by chromatography on diethylaminoethyl cellulose (*6*); Freund's complete adjuvant was used, and the rabbits were bled 3 weeks after the last injection. Specific antiserum reagent was prepared by completely absorbing the rabbit antiserums with normal rat serum, thus removing all antibodies against components of normal rat serum. Figure 1 shows the results of immunoelectrophoresis with unabsorbed and absorbed rabbit antiserum against acute-phase rat serum as the antigen; the only precipitin activity remaining after absorption is the antirat α_2 (acute phase)-globulin.

Samples of plasma perfusate were obtained at various intervals, frozen and thawed, heated for 30 minutes at 56°C, and centrifuged, and the clear plasma was dialyzed against isotonic saline for 48 hours at 4°C. An equal volume of the absorbed rabbit antiserums was added to dialyzed rat plasma, and the tubes were incubated for 2 hours at 37°C and left for 3 days at 5°C. The resulting immune precipitates were collected and washed three times with cold saline; they were then dissolved in 0.5 ml of 0.1*N* sodium hydroxide, and protein measurements were made on 0.1-ml portions of this solution by the method of Lowry *et al.* (*7*). The radioactivity in a remaining 0.2-ml portion of the solution was measured with a liquid-scintil-

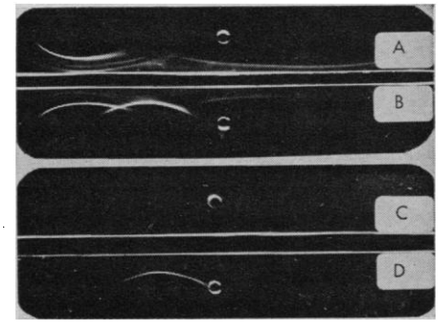

Fig. 1. Agar-gel immunoelectrophoretic patterns of normal and "acute phase" rat serums, illustrating the specificity of the rabbit α_2(acute phase)-globulin antiserums. Wells *A* and *C*, normal rat serum; *B* and *D*, "acute phase" rat serum containing α_2(acute phase)-globulin. Upper channel, unabsorbed rabbit antiserum prepared against a diethylaminoethyl-cellulose fraction of "acute phase" rat serum; lower channel, the same rabbit antiserum absorbed by normal rat serum.