Brief Definitive Reports

SPECIFIC HETEROLOGOUS ENHANCEMENT OF IMMUNE RESPONSES*

VI. PARTIAL PURIFICATION OF A NONSPECIFIC ENHANCING FACTOR FROM SUPERNATES OF ALLOGENEICALLY STIMULATED HUMAN LYMPHOCYTE CELL LINES

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Interactions between cellular components of the immune system are mediated, at least in part, by macromolecular chemical signals or lymphokines. This is true both for cellular immunity (cf. 1, 2 for reviews), and for the humoral immune response (cf. 3, 4 for reviews). The first indication of the latter was described by Hartmann in 1970 (5), who reported enhancement of the number of plaque-forming cells (PFC) against sheep erythrocytes (SRBC) in vitro when T cells from animals primed with horse erythrocytes were added with horse erythrocytes and SRBC to normal spleen cell cultures. In 1971, Dutton et al. (6) reported that the supernatant fluid from mixed lymphocyte cultures of allogeneic mouse cells restored the plaque-forming cell responses of mouse spleen cultures depleted of T cells by antiserum. At the same time Rubin and Coons (7) confirmed and extended Hartmann's findings using tetanus toxoid and other antigens for “nonspecific” enhancement. Such enhancement is due to a factor released by sensitized lymphocytes upon renewed contact with antigen. Subsequent reports have confirmed and extended these findings (8). The enhancing factor (EF) liberated from primed mouse spleen or thymus cells by antigen or allogeneic stimulation had a mol wt, in our hands, of approximately 75,000 daltons as measured by gel filtration on both dextran and polyacrylamide bead columns (8).

In this paper, we report the isolation of a soluble mediator from allogeneic mixtures of human lymphocyte cell lines which can nonspecifically enhance the humoral immune response of mouse spleen cells. This human enhancing factor

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(HEF) eluted from columns of Sephadex G-150 and BioGel P-150, with molecules of approximately 35,000 daltons.

Materials and Methods

Supernates were prepared from cultures of allogeneic mixtures of normal human lymphocyte cell lines. Cell lines RPMI 1788 (HL-A2, 7) and RPMI 4098 (HL-A3) (Associated Biomedic Systems, Inc., Buffalo, N. Y.) were maintained in medium RPMI 1640 supplemented with 10% fetal calf serum. Equal volumes of each cell line containing 2.5-5.0 X 10^8 viable cells/ml were mixed and cultured in spinner flasks for 24 h. The supernatant fluid was harvested by centrifugation (200 g, 10 min, 4°C) and stored at --20°C until fractionated and assayed for enhancing activity. Allogeneic supernatants were fractionated by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, N. J.) and BioGel P-150 (Bio-Rad Laboratories, Richmond, Calif.) on an 87 X 1.1-cm glass column by methods previously described (8).

Molecular weight calibration curves for the Sephadex and BioGel columns were produced by gel filtration of chemical markers of known molecular weight as described earlier (8). Supernates were fractionated and the column aliquots assayed for their ability nonspecifically to increase the anti-SRBC PFC response of normal mouse spleen cell cultures, i.e. for the presence of HEF, by methods used in previous reports (7, 8). Briefly, spleen cell cultures from normal 2-4-mo old DBA/2 female mice (Jackson Laboratories, Bar Harbor, Maine) were prepared by a modification of the method of Mishell and Dutton (7, 9) and were stimulated with 3 million SRBC on day 0. Filtered column fractions, diluted in fresh medium, were added to such cultures on day 2. Direct "19S" plaques against SRBC were enumerated on day 5. The degree of enhancement is expressed as the percent difference between the numbers of plaques produced in cultures that received diluted column fractions compared to the PFC response of similar cultures that received only the diluent. HEF was concentrated and purified from the allogeneic supernates by ammonium sulfate fractionation followed by gel filtration on Sephadex G-150, and polyacrylamide gel electrophoresis (8).

RESULTS

The results of a representative Sephadex G-150 gel filtration experiment are shown in Fig. 1. The column was calibrated with a mixture of four chemical markers of known molecular weight which were fractionated into four separate symmetrical peaks of activity (8). The elution pattern of these markers on Sephadex G-150 is indicated in Fig. 1 by the position of their respective molecular weights (arrows). When a 2.0-ml sample of a 24-h allogeneic supernatant was applied to the standardized G-150 column, we obtained two peaks of absorbance at 280 nm. Peaks of absorbance, due to the presence of fetal bovine serum components in the culture medium, were found at fractions 13 (28.6 ml of eluant) and 18 (39.6 ml of eluant), respectively (Fig. 1). Successive column fractions were assayed for the presence of the nonspecific human enhancing factor by addition of diluted aliquots to cultures of SRBC-stimulated, normal mouse spleen cells. As shown in Fig. 1, when column fractions were diluted 1:200 with complete medium and added to test cultures, a symmetrical peak of enhancing activity was found between fractions 19 and 25 (41.8 and 55.0 ml of eluant, respectively) with a peak of activity after fraction 22 (48.4 ml of eluant). Higher or lower dilutions of the peak fractions produced less enhance-
Fig. 1. Sephadex G-150 fractionation of the 24-h supernate from human lymphocyte cell lines. (● — ●), absorbance at 280 nm; (○—○), percent enhancement of PFC response (at 1:200 dilution) by an allogeneic supernate from a mixed culture of cell lines RPMI 1788 and 4098; (■—■), percent enhancement of PFC response (at 1:200 dilution) by the supernate of cell line RPMI 1788.

ment than the optimal dilution. This peak of activity was consistently found to elute with molecules with a mol wt of approximately 35,000 daltons. This was confirmed by gel filtration on a column of BioGel P-150. An enhancing molecule of similar size has also been isolated from the supernate of cultures of freshly drawn, normal human peripheral blood leukocytes cultured in the presence of tetanus toxoid.1 The leukocyte donor was shown to be primed to the toxoid antigen by finding antitoxin in his serum.

In contrast to the activity found in the supernatant fluid of cultures of mixed human lymphocytes, the culture medium from either cell line alone was devoid of detectable enhancing activity. (See Fig. 1).

The HEF from 100 ml of allogeneic supernatant was purified by a combination of preparative and analytical techniques (8). HEF-rich culture fluid was first treated with 50% ammonium sulfate. The enhancing activity in the 50% supernate was then precipitated by increasing the concentration of ammonium sulfate to 70%. The precipitate was redissolved in 5 ml of phosphate buffered

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1 Rubin, A. S. Unpublished data.
saline (PBS) and a 2.5-ml sample was fractionated on a column of Sephadex G-150. A single peak of enhancing activity was found to elute with molecules of 35,000 daltons. The three successive fractions embracing this peak from each of two column runs were pooled and concentrated to 1 ml by pervaporation. 25–150-µl samples were then subjected to disc electrophoresis on a step-gradient polyacrylamide gel containing 4.5, 6.0, and 8.0% acrylamide in Tris-sulfate buffer, pH 9.0 (8). The pattern of migration (150-µl sample) on a gel stained with amido black is shown in Fig. 2. An unstained gel, run in parallel, was cut into four sections as indicated (Fig. 2). The sections were homogenized in cold PBS, centrifuged to remove acrylamide, then dialyzed to remove toxic components (8). Enhancing activity was restricted to section B, which contained one faint band (arrow). The HEF recovered from this section was able to augment the PFC response of assay cultures by an average of 75% at a 1:200 dilution of the B supernatant.

**DISCUSSION**

Intensive study of long-term lymphoblastoid cell lines, derived from human peripheral blood, has shown that these cells can synthesize a variety of products including some mediators of cellular immunity (10). In this report, we have
demonstrated that mixtures of two such lymphocyte cell lines, which differ at the HL-A locus, are able to synthesize and secrete a cell-free soluble factor of 35,000 daltons which can nonspecifically increase the humoral immune response. Moreover, the activity of this HEF is effective, at least in vitro, across a species-specific barrier, i.e., it is capable of amplifying the productive antibody response of mouse B cells to a heterologous antigen. A factor of similar size and activity was specifically elicited from cultured normal human peripheral blood leukocytes from a tetanus toxoid-primed donor when the priming antigen was added to the culture medium.

In murine systems, specifically activated T lymphocytes have been shown to release soluble mediators which influence the humoral immune responses of syngeneic B lymphocytes (6, 8). The murine enhancing factor previously described was a protein of 75,000 mol wt which was elicited in vitro in a manner similar to that shown for HEF, i.e., by antigenic or allogeneic stimulation of primed thymus-derived cells (8). In the case of HEF, it is not unlikely that the human lymphocyte class which is triggered to release the factor is also thymus derived. However, we have not as yet shown this to be so. The human and murine enhancing factors differ in molecular weight, and also in electrophoretic mobility in polyacrylamide gels. HEF is 35,000 daltons in mol wt and migrates during gel electrophoresis to the section marked B in Fig. 2, while murine EF is larger (75,000 daltons) and has an electrophoretic mobility similar to that of the molecules in the band marked C (8). Despite their differences in size and charge density, these mediators quite clearly have similar functional effects when added to mouse spleen cell cultures 48 h after the induction of an in vitro immune response to a particulate antigen.

SUMMARY

The mixing of two histoincompatible human lymphocyte cell lines generated the release of a soluble factor which was capable of nonspecifically enhancing the in vitro immune response of normal mouse spleen cells against sheep erythrocytes. When active supernates were subjected to exclusion chromatography on Sephadex G-150 and BioGel P-150, the active principle eluted with molecules of approximately 35,000 mol wt. Column aliquots from similarly treated supernates from independent cultures of each lymphoid cell line were inactive. The human enhancing factor was concentrated and purified by ammonium sulfate fractionation, followed by Sephadex gel filtration and polyacrylamide gel electrophoresis.

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