Brief Definitive Report

AN ESTIMATION OF THE FREQUENCY OF PRECURSOR CELLS WHICH GENERATE CYTOTOXIC LYMPHOCYTES*

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The specificity of an immune response is generally regarded as the selection by antigen of precommitted precursor cells, followed by clonal expansion. The precommitment hypothesis is difficult to apply in the case of cell-mediated immunity where the frequency of responding cells is unexpectedly high. The frequency has been assessed by incorporation of [^{3}H]Tdr (tritiated thymidine) into DNA (1–3), removal of cells from the circulating pool (4), or the ability of cells to form virus plaques (5, 6). One problem encountered in these studies is the difficulty of correlating the number of cells that actually 'respond' with the number of precursors which become effector cells.

We have avoided this problem by segregating the effector cell clones with a polyacrylamide culture system of the type used previously to segregate the clones of antibody-forming cells (7). The experiments presented in this paper establish conditions in which the frequency of precursor cells in a CBA spleen able to generate a cytotoxic response against DBA mastocytoma cells has been measured.

Materials and Methods

Mice. 2–3-mo-old CBA/J and (CBA × DBA)F_1 mice were used throughout.

Culture System. Cells were cultured in polyacrylamide vessels as described previously (7). Each vessel contained 64 small depressions or 'dimples' into which the cells settled. After making the vessels in a perspex mold, they were washed, autoclaved, and stored at 4°C in sterile phosphate-buffered saline (PBS). Prewarming prevented any increase in the dimensions of the vessel during incubation which would cause a meniscus to form, with the subsequent loss of random settling of cells in the dimples.

RPMI 1640 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) contained 10% fetal calf serum (FCS) (Australian Laboratory Services), and 2-mercaptoethanol was added to a final concentration of 5×10^{-5} M. Untreated (CBA × DBA)F_1 spleen cells were used as stimulator cells, and a small number of CBA spleen cells were responder cells. The cells settled randomly into the dimples in the base of the acrylamide vessel. Cultures were grown in an atmosphere of 5% CO_2 in air.

Cytotoxicity Assay. The method used follows the procedure described by Brunner et al. (8). 5×10^6 DBA P815 mastocytoma cells were labeled with 100 μCi sodium ^{51}chromate (The Radiochemical Centre, Amersham) for 30 min at 37°C, washed three times, and diluted in RPMI 1640 containing 10% FCS. The contents of each dimple were mixed with 10^4 ^{51}Cr-labeled cells in 0.5 ml of medium. After centrifuging at 100 *g* for 3 min the tubes were incubated in 5% CO_2 in air at 37°C for 4 h. The contents of each tube were then thoroughly mixed, centrifuged at 500 *g* for 7 min, and a sample of the supernate was removed for scintillation counting. The ^{51}Cr released from the lysed mastocy-

* Supported by The Medical Research Council of New Zealand.

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toma cells was measured in a Packard Tricarb liquid scintillation spectrometer (model 3375, Packard Instrument Co., Inc., Downers Grove, Ill.) using the method described by Herscowitz and McKillip (9). The results were calculated as percent specific lysis using the formula described by Cerottini and Brunner (10).

$$\text{percent specific lysis} = \frac{^{51}\text{Cr released in presence of immune cells} - \text{spontaneous release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}} \times 100$$

Spontaneous ^{51}Cr release (8–16% after 4 h incubation) was estimated by incubating target cells alone or with normal lymphocytes, and maximum release (in the range 75–85%) was obtained by measuring the amount of ^{51}Cr released following the cycles of freezing and thawing. Cells from dimples that yielded more than 10% specific lysis were scored as positive. As this arbitrary percentage is a figure that is well above any apparently specific lysis due to errors in isotope counting or manipulation, the total calculation of the number of positive foci would be a conservative estimate.

Results

Kinetics of the Production of Cytotoxic Lymphocytes (CLs) in Acrylamide Dimples. 5×10^4 CBA spleen cells were cultured with 1.3×10^7 F_1 spleen cells, and individual dimples were assayed for cytotoxicity at various times. 64 dimples from duplicate cultures were assayed at each time. The number of foci were corrected for coincidence and are expressed in Fig. 1.

The kinetics of CL production are similar to those observed by other workers (11, 12). It is of interest to note that at the time when the maximum amount of cytolytic activity is found in in vitro responses, the maximum number of foci are detected in the polyacrylamide cultures (see Discussion).

The Frequency of Precursor Cells. It was assumed that the number of positive dimples is equivalent to the number of precursor cells in the responding

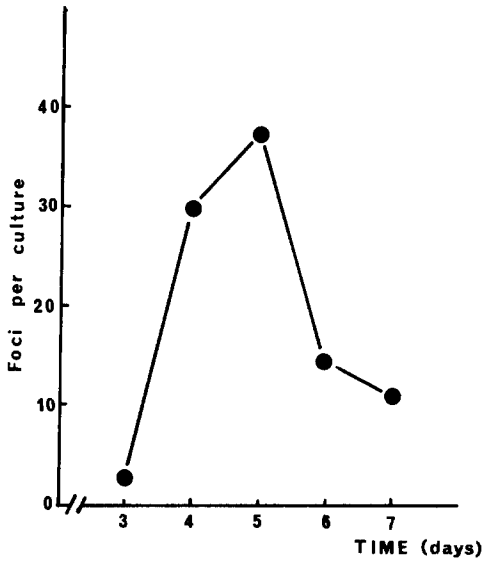

FIG. 1. Kinetics of CL production. 5×10^4 CBA spleen cells were cultured with 1.3×10^7 (CBA × DBA)F_1 spleen cells. On days 3, 4, 5, 6, and 7 the cells from half the dimples from duplicate cultures (total 64) were assayed for CLs. Results were corrected for coincidence, and the number of foci produced by F_1 alone were subtracted from each corrected figure.

population. To measure the frequency of precursors, cultures were set up with various concentrations of CBA spleen cells (10^4–1.25×10^5 cells). All cultures contained 1.3×10^7 F_1 spleen cells. Cells from individual dimples were harvested after 5 days and assayed for CLs. The data from three separate experiments are expressed in Fig. 2.

The results are plotted according to the method of Quintáns and Lefkovitz (13) in Fig. 2 A. There is a straight line relationship in the semilogarithmic plot of the results. The estimated frequency of CL focus-forming cells can be calculated from the concentration of cells which yields 37% negative dimples per culture. The frequency is 1 in 1,300 spleen cells. The straight line of Fig. 2 A does not extrapolate through the origin, and it was found that this was due to a small number of background foci. When F_1 spleen cells were cultured with no CBA cells there was a small number of definite positive foci of cytotoxic cells generated (11 foci per 1.3×10^7 cells on day 5). The range of specific lysis of cells from the positive dimples of these cultures ranged from 10 to 87%.

To obtain Fig. 2 B, the number of 'clones' in a culture was corrected for coincidence of foci as described previously (7). The number of foci of CLs which are generated in cultures of F_1 spleen cells was subtracted from each estimate, and the number of clones per culture is plotted against the cells cultured. The frequency of CL-forming cells was found to be 1 in 1,700 when corrected for the contributions of F_1 cells. 50 foci were generated in cultures containing 8.5×10^4 CBA spleen cells.

Discussion

The lack of techniques for the enumeration of antigen-sensitive T lymphocytes stimulated the development of the virus plaque assay (5), but the interpre-

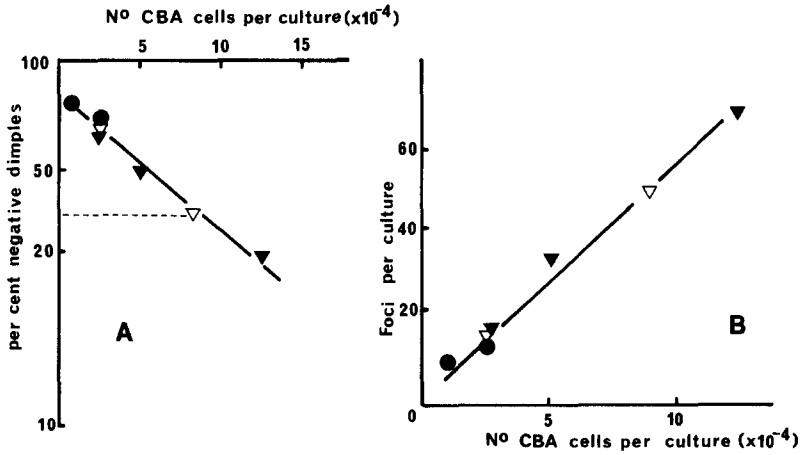

FIG. 2. Dose response relationships between CLs produced and the number of CBA cells cultured. Graded doses of CBA spleen cells were cultured with 1.3×10^7 (CBA × DBA) F_1 spleen cells. CLs were assayed in samples of cells taken from individual dimples after 5 days. There were 64 dimples per culture. (2 A) Results plotted in the manner of Quintáns and Lefkowits (14). Dotted line indicates the dose yielding 37% negatives. (2 B) The number of foci corrected for coincidence and background F_1 foci subtracted. Three separate experiments indicated by different symbols.

tation of results using this assay is complicated by the fact that cell proliferation and the production of cytotoxic lymphocytes may be dissociated from one another (14). The use of the polyacrylamide culture system has allowed the segregation of foci of CL so that within the limits of the ^{51}Cr-release assay, the number of precursors of CLs can be measured. Since, unlike Wagner and Feldman (11), we did not find that mitomycin C-treated cells were superior to untreated F_1 cells, F_1 cells have been used in all experiments.

The frequency of foci of CLs under the conditions of the polyacrylamide cultures has been derived using the rationale of Quintáns and Lefkovitz (13) in Fig. 2 A. If the number of foci derived from F_1 cultures is subtracted as in Fig. 2 B then the frequency is 1 in 1,700. This figure may be compared to the frequency of 'responding' cells which has been estimated at 1–12% according to various estimates (1, 2, 3, 15). Our estimate of 1 in 1,700 must represent the minimum frequency, as the plating efficiency of the cultures is unknown and the method depends on the sensitivity of the ^{51}Cr-release assay. In addition to the frequency estimation, an interesting feature of these results is the small number of foci in cultures of F_1 cells. Although the generation of CLs has been demonstrated to be derived from θ-positive lymphocytes (12 and unpublished data), we have yet to delineate the nature of the apparently syngeneic cell killing. No mastocytoma cells were included in the cultures, so that if the CLs are directed against antigens that are unique to the tumor cell, the foci were generated in the absence of specific stimulation. Of the explanations for this phenomenon we could consider firstly, the 'polyclonal' type of response induced by mitogenic factors in the fetal calf serum or secondly, the removal of suppressor cells. The segregation of precursors may also be diluting out suppressive influences.

The dose response relationship in Figs. 2 A and B is consistent with the foci being derived from the clonal expansion of single precursors although we have not formally demonstrated this fact.

The peak of the cell-mediated immune response may be defined as that time when the maximum number of effector cells are produced. If the response consists of the early activation of all precursors, followed by clonal expansion, the maximum number of foci might be expected to occur before the peak of the total response. The kinetics of antigen-induced activation of immune cells suggest that it is an exponential process (for example, [16]). Bloom et al. (17) have found a linear increase of virus plaque-forming cells in a cell-mediated response and have interpreted their results as demonstrating that the increase is due to the rate at which cells progress from a normal resting stage to an active dividing state. As described previously for antibody production (7), the data in Fig. 1 are consistent with the progressive activation of precursors with, presumably, the exponential increase of effector cells within each clone.

The segregation of specific effector cells allows an estimation to be made of the number of precursors of CLs. From a consideration of published data on the frequency of responding cells (cells with initiator function) and our data on the frequency of precursor cells of CLs, the effector cell precursors may be regarded as a subset of the total responding population. Wilson (19) has discussed the alternative schemes that have been devised to account for the range of responses by histocompatibility antigen-reactive cells. The implications of the precommitment hypothesis may be accommodated by assuming that CLs are generated by

the clonal expansion of a subset of precursors which appear with low frequency. In the absence of data on the affinity parameter of the responding foci, it is difficult to assign any value to what might be regarded as a 'low frequency'. Our figure of 1 in 1,700 is the minimum frequency and is related to the total number of nucleated spleen cells. It should be possible to extend the clonal segregation technique to determine whether initiator and effector populations can be separated in the same culture.

Summary

The cell-mediated immune response has been generated in vitro with a polyacrylamide culture system which allows the segregation of foci (clones?) of cytotoxic lymphocytes. Using the method of limiting dilutions, the frequency of precursor cells in CBA spleen cells able to generate a cytotoxic response against DBA mastocytoma is estimated at 1 per 1,700 cells.

Received for publication 15 December 1975.

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