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RECEPTOR ACTIVITY OF SORTED LYMPHOID CELLS WITH A THYMIC REGULATORY FACTOR

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SUMMARY.

A small molecular-weight regulatory factor from the thymus has been shown to enhance both cell-mediated and humoral immune responses. In this study, the relative binding capacity of the factor to thymus-dependent and bone marrow-dependent lymphocytes was measured directly. Spleen cells were separated using an automated, multiparameter, cell sorting instrument. Receptor activity for the iodinated thymic protein of each population was determined. Based on a number of criteria, thymus-dependent cells exhibited a greater affinity for the regulatory protein than did the bone marrow-dependent cells. These results indicate that the mechanism of action of regulatory control is via receptor-ligand interactions of specific subpopulations of lymphoid cells with a humoral factor.

INTRODUCTION.

Thymosin has been shown to enhance cell-mediated or T-cell[1] responses in mouse systems *in vivo* (1) as well as *in vitro* (2). Our previous findings (3) indicate that humoral or B-cell[2] immune responses are amplified *in vitro* by a thymic protein which is preparatively analogous to a fraction of crude thymosin. The precise mode of action of this regulatory factor is not known and the responsive lymphoid cell population has not yet been identified. The present investigation was undertaken to determine whether either (T or B) or both lymphoid cell populations possess specific receptors for this factor.

Our preliminary (unpublished) studies, using mechanical means of cell separation such as adherence to nylon wool, gradient methods and selective inactivation by specific antisera, indicate that the presence of T-cells is a requisite for binding the regulatory protein. To substantiate these findings, an automated, multiparameter, cell-sorting instrument was employed, and the

[1] T-cells, thymus-dependent lymphoid cells

[2] B-cells, bone marrow-dependent lymphoid cells

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receptor activity of separated populations of lymphoid cells was measured. Based on specific criteria, T-cells exhibited greater affinity for the regulatory factor than did B-cells. Thus, while both humoral and cell-mediated immune responses are subject to regulation by factors from the thymus (4), the mechanism of action of this regulatory control is apparently initiated by receptor-ligand interactions on the surface of T-cells. In addition, the regulatory effect is markedly augmented by elevating cyclic nucleotide levels in the cell environment, which is consistent with other studies (3,4,5) of mechanisms of T-cell action.

MATERIALS AND METHODS.

Preparation and Iodination of the Thymic Protein. The thymic regulatory factor, crude bovine fraction three, was prepared according to the method of Goldstein and his associates(2). Biological activity was measured by a plaque-forming cell assay and by determining hemagglutination titers (3). The preparation was chromatographed on G-50 Sephadex (Pharmacia) columns and peak fractions corresponding to molecular weights of 10-15,000, when compared to standards on acrylamide gel electrophoresis, were pooled, concentrated and on a per weight basis exhibited a 10-fold increase in biological activity[1]. This protein was iodinated by a modified method of Marchalonis et al. (6,7) using lactoperoxidase (Sigma) and ^{125}I-sodium iodide from New England Nuclear Corporation. The iodinated BF3 was separated from unreacted sodium iodide and from lactoperoxidase by chromatography on G-75 Sephadex columns. To check the identity of the iodinated protein, an aliquot was co-chromatographed with the original BF3 and the eluted counts were compared to the absorbance profile at 280 nm wavelength.

Membrane Labelling of Lymphoid Cells. Receptor-binding experiments were conducted using spleen cells from CBA-J and BDF/1 mice. Culture media and components were purchased from Grand Island Biological Co, (N.Y.). Fluorescein isithiocyanate[2]-labelled antimouse immunoglobulins G and M[3] were purchased from Behring Diagnostics. The sera were heated to 56°C to inactivate complement and were repeatedly absorbed against liver acetone powders to eleminate nonspecific reactivity. Mice were killed by cervical dislocation, spleens removed and cells separated by gently pressing through a number 60 mesh (250 u size openings). The cells were collected into medium which contained 10% fetal calf serum and 10^{-6}M mercaptoethanol. The cells were washed and pelleted and layered over 34% Ficoll and centrifuged at 400xg for 15 minutes using an IEC PR 6000 centrifuge. The white cells at the interphase were collected and washed and their viability measured. Staining of cell membranes with FITC-labelled antiserum was ascertained by viewing preparations with a Zeiss UV microscope equipped with darkfield illumination.

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1 This preparation will subsequently be referred to as BF3.

2 FITC, fluorescein isthiocyanate

3 IgG, immunoglobulin G; IgM, immunoglobulin M

Multiparameter Cell Sorting and Analysis. Cell sorting based on light-scatter and fluorescence properties of FITC-labelled mouse cells was carried out essentially as described by Mullaney and his associates (8,9). Briefly, approximately 10^6 cells were introduced into a dual-sheath flow chamber at a rate of 1000 cells /second from a pressurized reservoir. Cell volume and cell fluorescence were sequentially measured on each cell, using the Los Alamos Scientific Laboratories flow chamber whichhas incorporated in it a Coulter volume-sensing device. Cell fluorescence was quantitated as each cell passed through an elliptically focused, 488-nm wavelength laser beam. Following cell analysis, the cell stream was broken into droplets by the action of a piezoelectric transducer. Each cell was thus placed in a droplet. Processed cell volume and fluorescence signals trigger the cell sorting by comparison of the amplitude of each signal with preset standards. Droplets containing cells and meeting selected criteria were charged and deflected by a static electric field into a collection vessel.
Binding of ^{125}Iodine-BF3 to Sorted Cells. In addition to the unsorted group, cells were sorted as a brigthly fluorescing and a dimly fluorescing group. These three groups of cells, as well as a control sample containing medium alone, were incubated at 37°C in the following reaction mixture: 500 ul of cells (generally 10^6 to 10^7 cells/reaction); 100 ul of fetal calf serum and 10^{-6}M mercaptoethanol, 10^{-6}M cyclic AMP[1], and 50 ul ^{125}I-BF3 (containing aproximately $5x10^6$ cpm and 20 ug protein). After incubation for selected time intervals, 50 ul aliquots were withdrawn and added to a "Stopping" solution which contained unlabelled BF3 in 0.1 M KI. Binding of BF3 to cells was determined after washing them in cold medium on Millipore filters and counting the radioactivity. Binding of BF3 to cell membranes and to proteins of the soluble fraction was determined after cell homogenization and subfractionation. Binding of BF3 to the soluble fraction was measured after precipitation with trichloroacetic acid and filtering as described above.

RESULTS.

Characterization of the Iodinated Thymic Factor. Our partially purified thymic protein exhibited identical chromatographic characteristics before and after iodination. These results are shown in Figure 1. and indicate that the original and the iodinated proteins have superimposable chromatographic profiles. Biological activity of the thymic protein could be shown to increase plaque formation and hemmagglutination in cultured spleen cells(3). Optimum amplification of these immune responses occured when the culture medium contained 10^{-6}M cyclic AMP. The receptor binding study was conducted under the same conditions. Thymic factors and cyclic nucleotides are apparently cooperatively involved in augmenting lymphoid cell responses (5,10,11) and point to the role of these agents as effectors of stimulus responses in these cells.

Sorting of FITC-labelled Cells. After separation and exposure to the antiglobulin preparation, fluorescein-labelled cells accounted for 30 to 60% of the

[1] cyclic AMP, cyclic 3',5'-adenosine monophosphate

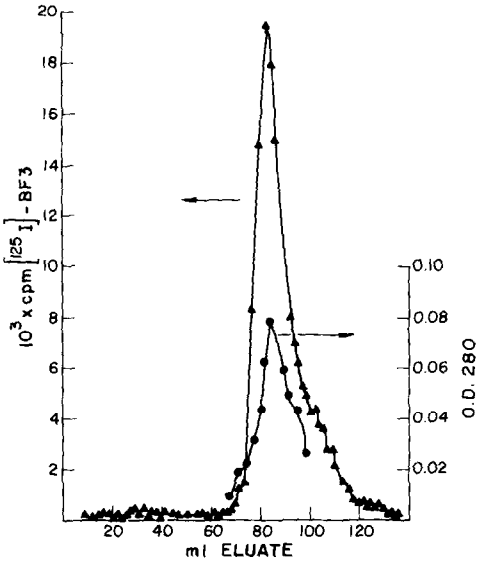

Figure 1. G-50 Sephadex Chromatography.
Purified and iodinated BF3 was co-chromatographed with the original sample and the radiocative (due to ^{125}I-BF3) and the absorbance profiles (in the UV range, 280 nm) were compared. Blue dextran and RNAse were eluted at 40 and 75 ml respectively.

total when counted in randomly selected high-power fields. Cytotoxic effects were observed in antiserum concentrations greater than 20% and the best results were obtained with a 12% FITC anti-IgG solution in the reaction medium. Viability under these conditions was>90%. Lymphoid cells which were membrane-stained with FITC-labelled antimouse IgG or IgM serum, were then subjected to sorting procedures as defined by Mullaney and associates (12, 13) and outlined in Methods. Cell sorting results of a number of consecutive experiments were consistent and the procedure adopted for receptor studies was one in which the least amount of nonspecific fluorescence was present and which contained the fewest red blood cells and dead cells.

Typical fluorescence and light-scattering properties of the cells are shown in Figure 2. The distribution of fluorescent cells is represented on the left side of the graph, while the right side shows the distribution of light scattering properties. Investigations using our instruments have shown that live

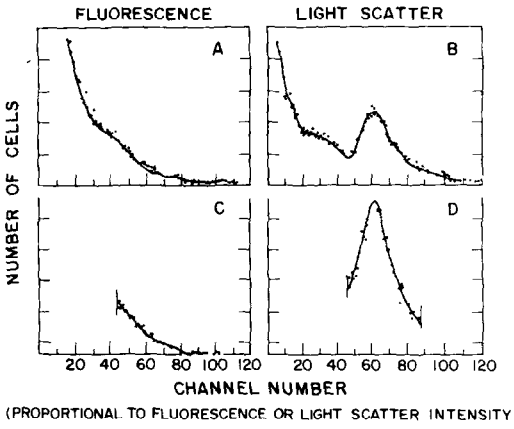

Figure 2. Graphic representation of fluorescence and light scattering propeties of sorted cells. Panel A shows the fluorescence profile; panel B the light scattering; Panels C and D show the portion of the distributions which were selected for sorting.

cells scatter more light than do dead cells and debris. Similar findings are reported by other investigators (14). The main peak, in channel 60 of Figure 2B consists of live cells and the shoulder to the left of dead cells and cellular debris. Thus, most dead cells are excluded from the sorted populations, since their scattering intensities are in a different range and are electronically "gated out" from the sorted cell fractions. Figures 2C and 2D show the portion of the distributions which were selected as the regions from which cells were sorted. Three electronic gates (shown as vertical lines) were established prior to sorting. In order for a cell to be selected, two requirements had to be fulfilled: its fluorescence signal either had to be greater (such as the data in Figure 2C) or less than the window value shown in Figure 2C and its light-scatter signal had to fall within the window shown in Figure 2D. These requirements resulted in fractions of sorted cells that were viable and immunoflrorescently "positive" or immunofluorescently "negative". Consequently, brightly fluorescing B-cells were sorted into one collection vessel while less brightly fluorescing T-cells were sorted into another container. The relative ratios of

TABLE 1.

Binding of ^{125}-BF3 to Spleen Cells

Cell group	mg protein per reaction	nmoles BF3 bound	CPM in sol. fr.
A (unsorted)	0.42	7.56	5828
B (sorted right) *	0.32	2.35	3448
C (sorted left) **	0.12	20.44	491
D (medium alone)	0	0	0

Incubation was carried out for 60 minutes in medium containing 0.57 mg/ml BF3, 10^{-6}M mercaptoethanol, and 5 x 10^{-6}M cyclic AMP. nmoles of BF3 bound are reported per mg protein.
* strongly FITC stained cells
** weakly FITC stained cells

sorted cells in our experiments correspond well with those obtained by other investigators using similar instrumentation (15, 16).

Receptor-BF3 Binding Activity. Unsorted cells, sorted T-cells and sorted B-cells, have different affinities for BF3, as indicated by the results shown in Tables 1 and 2. Table 1 shows the extent of ^{125}I-BF3 binding after a 60 minute incubation with the various cell populations. While both group A, unsorted cells, and group B, mainly B-cells, form some, transient association with BF3, T-cells show the most stable and marked binding capability. These findings are in agreement with the results of *in vivo* and *in vitro* experiments of other investigators (16,17) which indicate that the responsive population of cells to a thymic regulatory protein are those involved in cell-mediated immunity (the T-cells).

The data in Table 2 represent the time course of BF3 biding to separated lymphocyte populations of two different experiments and indicate that while short-term binding of BF3 to B-cells might occur under conditions of high BF3 labelling as in experiment I, it decreases sharply over time (column B), while a conventional pattern of receptor-ligand binding activity holds for T-cells. Binding appears specific and increases linearly with time for approximately 10

TABLE 2.

Time Course and Kinetics of BF3 Binding to Lymphoid Cell Membranes.

Experiment number	Time (min)	nmoles BF3 bound (10^6 cells)		nmoles BF3 bound (10^6 cells)/min.	
		B	C	B	C
I	2.5	2.05	1.45	0.82	0.58
II	5.0	0.40	1.20	0.16	0.48
I	7.5	3.28	7.40	0.44	0.99
II	9.2	2.60	9.20	0.35	0.99
I	15.0	1.46	14.13	0.10	0.94
II	"	2.65	11.25	0.18	0.75
I	25.0	3.46	14.31	0.14	0.57
II	"	1.95	13.80	0.08	0.55

Experimental conditions and cell group designations as described in Table I. Counts per reaction mixture were less in Experiment II than in Experiment I, due to isotope decay (I- 3.12 x 10^6 CPM; II- 1.95 x 10^5CPM).

to 20 minutes, after which a levelling off occurs. Binding to the B-cells on the other hand appears less specific and decreases rapidly with time. These results are shown in Figure 3 The counts associated with the cell membrane fraction after removal of soluble components, indicate that BF3 binding is greater to T-cell membranes. In addition, binding is linear in the first 10 minutes, which is compatible with conventional receptor-ligand kinetics.

When counts due to ^{125}I-BF3 were measured in the soluble fraction of cells (last column, Table 1), B-cells and unsorted cells showed greater binding than did T-cells. This may be the result of a pinocytotic process rather than specific cell-surface and receptor-ligand interactions and may account for the BF3 associated with B-cells. On the other hand, counts in the mebrane fractions of T-cells are significantly greater than in the soluble fractions and are more likely to be a representation of membrane receptor activity.

DISCUSSION

This study provides the first direct demonstration of binding of a thymic

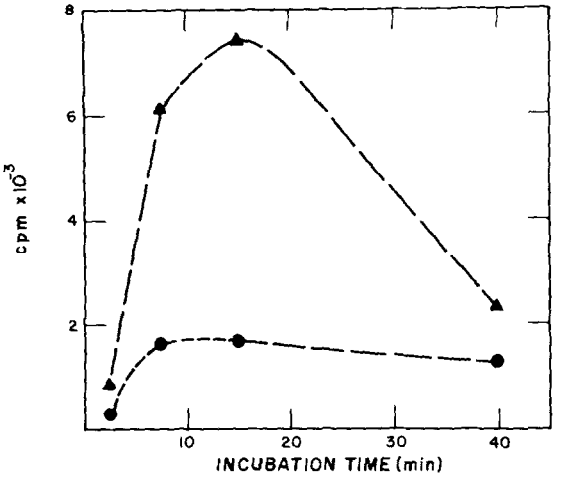

Figure 3. Time course of ^{125}I-BF3 uptake of sorted spleen cells. The CPM incorporated into the T-cells (▲---▲) as compared to the CPM incorporated into the B-cells (●----●) are plotted against time.

protein to a specific lymphocyte population. While numerous indirect methods have been employed to show T-cell interactions with thymic factors, use of a cell sorting instrument provides sufficient quantities of a purified cell population to carry out biochemical determinations in viable cells. Our study has shown that T-cells bind the iodinated thymic protein and this binding is associated with the protein-containing membrane fraction. Definitive experiments are yet to be carried out to demonstrate that isolated membrane proeins have receptor activity in vitro.

The results of a number of studies (11,17) implicate T-cells as the lymphocyte population which is responsive to regulatory control by a circulating factor from the thymus. This factor has been shown to stimulate immune responses (3,10) and its action is apparently related to changes in cyclic nucleotide levels (18). While the mode of regulatory action of this factor is not known, a likely mechanism may involve the recognition and binding of this protein to a specific T-cell subpopulation (possibly helper cells or their precursors). If the events were to follow established patterns of cyclic AMP-mediated regulatory

action, then specific acidic protein phosphorylation would lead to differentiation of these cells into a functionally competent group.

The availability of instrumentation used in this study provides an opportunity to address the above hypothesis on an experimental basis. Further studies wherein specific cell populations are obtained from the mixture of cells present in a tissue can now be carried out to measure specific cellular events. Systems for peripheral blood lymphocytes (19,20) and for cells from cervical smears (13) have been studied, indicating that cells from various mammalian sources can be successfully sorted and utilized for biochemical studies.

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REFERENCES

1. Goldstein, A.L., Asanuma, Y., Battisto, J.R., Hardy, J., Quint, M.A., and White, A.(1970). J. Immunol. 104, 359.
2. Bach, J.F., Dardenne, M., Goldstein, A.L., Guha, A., and White, A.,(1971). Proc. Natl. Acad. Sci. 68. 2734.
3. Mednieks,M.,I. and Juras, D.S.,(1975). Immunol. Comm.,4, 99.
4. Kook, A.I. and Trainin, N.J.,(1974). J. Exp. Med., 139, 193.
5. Marchalonis, J.J. and Smith, P., (1976). Aust. J. Exp. Biol. and Med. Sci., 54,1.
6. Marchalonis, J.J., Cone, R.E. and Atwell, J.,(1972). J. Exp. Med., 135, 956.
7. Marchalonis, J.J. and Cone, R.E.(1973). Transplant. Rev., 14,3.
8. Mullaney, P.F., Crowell, J.M., Salzman, G.C., Martin, J.C., Hiebert, R.D. and Goad, C.A. (1976). J. Histochem. Cytochem., 24, 298.
9. Mullaney, P.F., Steinkampf, J.A., Crissman, H.A., Cram, L.S. and Holm, D.M. (1974). In "Laser applications in Medicine and Biology." p.151. Plenum Press.
10. Bach, J.F., Dardenne, M., Plau, J.M. and Rosa, J., (1976). Comptes Rendus, 283, 1605.
11. Naylor, P.H., Sheppard, H., Thurman, G.B. and Goldstein, A.L., (1976). Biochem. Biophys. Res. Comm., 73,843.
12. Mullaney, P.F. and Dean, P.N., (1970). Biophys. J., 10, 764.
13. Salzman, G.C., Crowell, J.M., Hansen, K.M., Ingram, M. and Mullaney, P.F., (1976). J. Histochem. Cytochem., 24, 308.
14. Loken, M.R., Sweet, R.G. and Herzenberg, L.A., (1976). J. Hostochem. Cytochem., 24, 284.
15. Fathman, C., Small, M., Herzenberg, L.A. and Weissman, J.L., (1975). Cellular Immunol., 15,109.
16. Marchalonis, J.J., (1969). Biochem. J., 113,299.
17. Bach, M.A. and Beaurain, C., (1976). Ann. D'Immunol., 127,967.
18. Bosing-Schneider, R. and Haug, M., (1976). Cellular Immunol., 27,121.
19. Cram, L.S., Gomez, E.R., Thoen, C.O., Forslund, J.C. and Jett, J.H., (1976). J. Histochem. Cytochem., 24,383.
20. Cassidy,M., Yee,C.,and Costa, J.J. (1976). J. Histochem. Cytochem., 24,373.