Brief Definitive Report

LYMPHOCYTE ACTIVATION BY MONOVALENT FRAGMENTS OF ANTIBODIES REACTIVE WITH CELL SURFACE CARBOHYDRATES*

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The critical initial event in stimulation of lymphoid cells by specific ligands such as antigens, and relatively nonspecific ligands such as antibodies and lectins, is the binding to appropriate receptor sites on the cell surface. There is considerable evidence to suggest that in order for ligands to activate lymphocytes, they must be at least divalent. This view derives principally from studies in which monomeric fragments of antibodies to immunoglobulins or other lymphocyte surface determinants failed to stimulate (1–4). Among these antibody reagents directed against various cell surface components, only Fab fragments of goat antirabbit immunoglobulin allotype antibodies were reported to be mitogenic (5).

We have recently reported (6) the isolation and characterization of antibodies reactive with cell surface carbohydrates (carbohydrate-specific immunoglobulin [CS-Ig]). In addition, we found that the CS-Ig fractions from normal chicken serum were mitogenic for mouse splenic lymphocytes. The availability of a mitogenic reagent that can be made monovalent provides a unique opportunity to test the requirement for multivalency in lymphocyte stimulation involving interaction with carbohydrate-containing glycoproteins and glycolipids. In the present communication, we report evidence indicating that the monovalent fragment of CS-Ig (chicken) is as potent a mitogen as the native divalent antibody. We consider several mechanisms to account for the stimulation of lymphocytes by univalent ligands.

Materials and Methods

CS-Ig (chicken) was isolated from normal chicken serum (Grand Island Biological Co., Grand Island, N. Y.) by affinity chromatography on Sepharose covalently coupled with the glycoprotein fetuin (6). Fab′ fragments of CS-Ig (chicken) were obtained by digestion of the immunoglobulin molecule with pepsin (Worthington Biochemical Corp., Freehold, N. J.) in 0.1 M sodium acetate, pH 4.5, using an enzyme to protein ratio of 1% (7). After 12 h at 37°C another sample of enzyme was added. The digestion was terminated by adjusting the pH of the solution to 8 followed by dialysis against phosphate-buffered saline (PBS). Parallel experiments were performed on normal chicken immunoglobulin isolated from chicken serum by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography (8).

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CS-Ig and Fab'-CS-Ig were succinylated by following the procedure described (9) for the derivatization of concanavalin A (Con A). Succinylation was performed at 4°C for 1 h using a reagent to protein ratio of 1:3 followed by dialysis against PBS and a second derivatization at room temperature for 90 min (9).

Con A was isolated as described previously (10). Cell culture media, antibiotics, and fetal bovine serum were from Microbiological Associates, Inc., Bethesda, Md. Lymphocytes were obtained from spleens of NCS mice (The Rockefeller University, New York).

Mitogenesis assays were conducted by measurements of [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., 1.9 Ci/mmol) incorporation and blast transformation. The detailed conditions are described elsewhere (11). Procedures for polyacrylamide gel electrophoresis under dissociating (12) and nondissociating (13) conditions have also been described.

Results

As we had reported previously (6), CS-Ig (chicken) showed mitogenic activity for mouse spleen cells. The mitogenic response of these cells as assayed by [^{3}H]thymidine incorporation was weak as compared to that observed using the lectin Con A. Quantitation of blast transformation showed that a lower percentage of cells responded to the antibody reagent than to the lectin (Table I). Moreover, the fact that the average number of grains per labeled blast cell was the same in Con A-treated and CS-Ig-treated cultures indicated that the activated cells all synthesized DNA to approximately the same extent, independent of the nature of the mitogenic agent. In any case, control experiments showed that the weak but reproducible response was not due to antigenic stimulation, inasmuch as normal chicken IgG did not stimulate.

In order to determine whether the monovalent Fab fragment of CS-Ig retains its capacity for stimulation, the CS-Ig was digested with pepsin. It has been shown previously that pepsin treatment of chicken immunoglobulins results directly in the formation of Fab' fragments (14). Pepsin digestion of CS-Ig therefore converted the native molecule into a new species corresponding to the Fab' fragments obtained by parallel treatment of normal chicken immunoglobulin (Fig. 1 *a*). This product, designated Fab'-CS-Ig, showed no detectable contamination by divalent antibody on polyacrylamide gels. Moreover, when the agglutinating activities for pig erythrocytes were compared for CS-Ig and its Fab' fragment, it was found that the native antibody agglutinated cells at 3 μg/ml, whereas the pepsin fragment did not agglutinate even at 2 mg/ml.

TABLE I

*Comparison of the percentage of cells responding to Con A and to CS-Ig**

	cpm	Labeled blast cells	Blast cells	Average no. of grains/blast cell
		%	%	
Control	2,075	0.50	0.50	ND
Con A	45,000	18.80	22.53	102.5
Normal chicken IgG	2,900	0.65	1.25	ND
Fab' chicken IgG	2,100	0.45	0.68	ND
CS-Ig (chicken)	12,400	7.60	8.77	89.0
Fab' CS-Ig (chicken)	15,860	6.16	6.80	104.9

* The data represent the average of duplicate determinations. The concentrations of mitogenic agents used in these experiments were 3 μg/ml for Con A and 120 μg/ml for the antibody reagents.

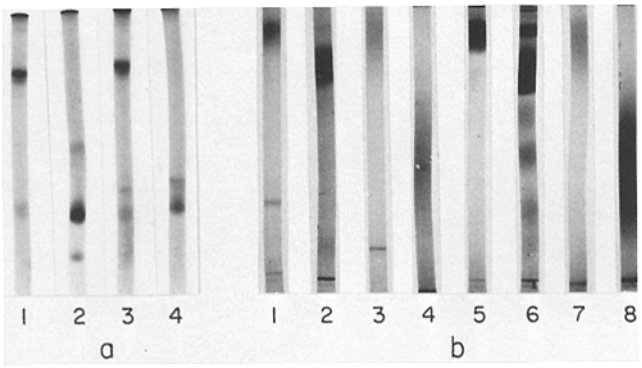

FIG. 1. *(a)* Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (12) of: (1), CS-Ig (chicken); (2), Fab'-CS-Ig (chicken); (3), normal chicken IgG; and (4), Fab' chicken IgG. *(b)* Polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 9.3 (13), of: (1), CS-Ig (chicken); (2), succinylated CS-Ig (chicken); (3), Fab'-CS-Ig (chicken); (4), succinylated Fab'-CS-Ig (chicken); (5), normal chicken IgG; (6), succinylated chicken IgG; (7) Fab' chicken IgG; and (8) succinylated Fab' chicken IgG.

Fab'-CS-Ig was as potent a mitogen for mouse spleen lymphocytes as the native immunoglobulin molecule (Fig. 2 and Table I). Under the conditions of the assay, both CS-Ig and its Fab' fragment showed maximum stimulation at concentrations of about 100 μg/ml. Moreover, the increase in [^{3}H]thymidine incorporation as a function of concentration was approximately the same for the two proteins. The final level of the response to Fab'-CS-Ig was slightly higher than the response to an equal weight of CS-Ig.

In order to avoid the possibility that the Fab'-CS-Ig fragments might form aggregates in solution and on the cell surface, the fragments were succinylated to increase their net negative charge. Parallel chemical modification was also performed on the native CS-Ig as well as on normal chicken immunoglobulin and its Fab' fragment. Succinylation was assessed by the altered electrophoretic mobility in polyacrylamide gels (13) under conditions that separated molecules by differences in ionic charge. Treatment with succinic anhydride changed the gel migration patterns of CS-Ig and Fab'-CS-Ig in a fashion similar to that observed with their normal chicken immunoglobulin counterparts (Fig. 1 *b*). The mitogenic response of mouse spleen cells to both succinylated CS-Ig and succinylated Fab'-CS-Ig showed no significant change from that obtained with the unmodified antibody and its Fab' fragment (Fig. 2).

Discussion

There is considerable evidence to suggest that the stimulation of lymphoid cells by ligands reactive with cell surface components requires multipoint attachment and cross-linkage of certain cell surface receptors. This view derives principally from studies demonstrating that monomeric fragments of antibodies to lymphocyte surface determinants fail to stimulate proliferation of the cells. These studies include the stimulation of rabbit lymphocytes by antiimmunoglobulin antibodies (4), the stimulation of human and mouse lymphocytes by antilymphocyte sera (1–3), and the stimulation of cultured L cells by anti-L-cell

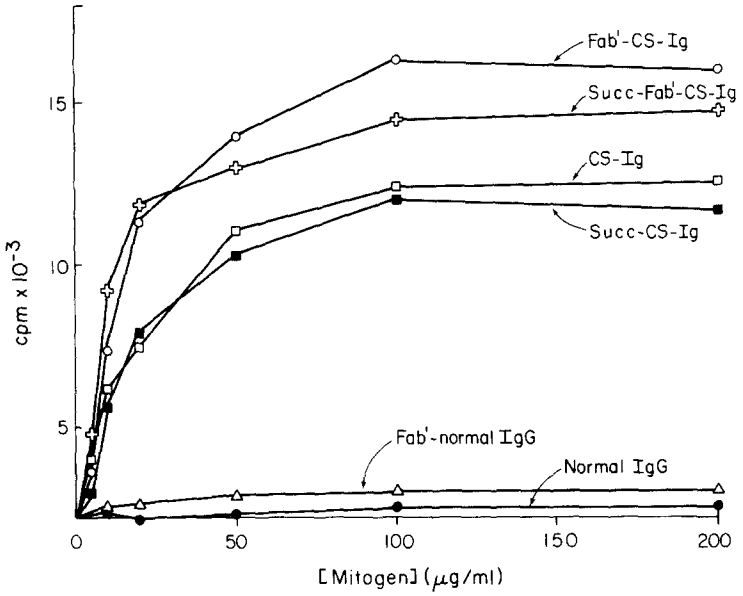

FIG. 2. Dose-response curve of the incorporation of [^{3}H]thymidine in the stimulation of mouse spleen cells by CS-Ig (chicken), Fab'-CS-Ig (chicken), and their succinylated (Succ) products. The response of the cells to normal chicken IgG and Fab' fragment is also presented.

antibodies (15). In contrast, however, Sell (5) found that Fab fragments of antirabbit immunoglobulin allotype antibodies were mitogenic.

The present experiments were designed to test the receptor cross-linkage hypothesis for the mechanism of lymphocyte stimulation using antibodies reactive with cell surface carbohydrates. The results showed unequivocally that the univalent Fab' fragment of CS-Ig was just as mitogenic for mouse splenocytes as the divalent native antibody. The mitogenic activity of the monovalent Fab' preparation is probably not due to small amounts of contaminating CS-Ig, for gel electrophoretic studies showed little contamination by material with an electrophoretic mobility corresponding to 150,000 daltons (Fig. 1). In addition, the increase in the cellular response as a function of mitogen concentration was approximately the same for the native antibody and its Fab' fragment (Fig. 2). Finally, agglutination assays using pig erythrocytes indicated that the Fab' preparation contained at most 0.1% of the divalent material.

These findings suggest, therefore, that univalent Fab'-CS-Ig can stimulate cells and that it may bypass the requirement for receptor cross-linkage on the cell to which it binds. In support of this conclusion, we have recently found[1] that another univalent saccharide-specific ligand, a monovalent derivative of Con A, was just as mitogenic for mouse and human lymphocytes as divalent succinyl-Con A and tetravalent native Con A. It has also been found that the B-chain monomer of ricin is weakly mitogenic for human lymphocytes (16). Although the

[1] Fraser, A. S., J. J. Hemperly, J. L. Wang, and G. M. Edelman. 1975. A monovalent derivative of concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* In press.

mitogenic activity of the monovalent preparations of both lectins may possibly be accounted for by contaminating dimers and aggregates, the results obtained with succinylated Fab'-CS-Ig cannot be accounted for in this fashion. It appears, therefore, that for saccharide-specific ligands, receptor cross-linkage may not be a stringent requirement for lymphocyte stimulation.

One possible mechanism of stimulation by univalent Fab'-CS-Ig involving receptor cross-linkage is the reaggregation of the monovalent molecules in solution or on the cell surface to become effectively multivalent. Although extensive electron microscope studies may be required to rigorously rule out this possibility, the fact that the succinylated Fab' fragments, which are subject to charge repulsion, showed similar mitogenic properties makes it appear unlikely. In an alternative mechanism, Fab'-CS-Ig molecules may be cytophilically adsorbed on the surface of macrophages which then present them in a multivalent form to the responding cells. Recent experiments (17, 18) demonstrating an absolute requirement for macrophages in the stimulation of certain subpopulations of lymphocytes by lectins and other reagents are in accord with the idea that the macrophages may play a key role in mitogen-lymphocyte interactions.

The hypothesis implicating the participation of another cell in concentrating, modifying, and presenting the mitogen to a responding cell may also include lymphocyte-lymphocyte interactions through a mitogenic agent. It has been proposed previously (19, 20) that in the stimulation of lymphocytes by $NaIO_4$, periodate-treated cells may present "foreign surface determinants" that activate other cells in a pseudo mixed lymphocyte reaction. Recent experiments (21) suggest that mitogenic stimulation by Con A may also occur through an indirect mechanism, analogous to a mixed lymphocyte reaction. Interpreted in light of these findings, the stimulation of lymphocytes by monovalent Fab'-CS-Ig and monovalent derivatives of Con A suggests that the receptor cross-linkage necessary for cell activation may be achieved by indirect mechanisms of mitogen presentation as well as by the multivalent properties of the mitogenic agent itself.

Summary

Antibodies reactive with cell surface carbohydrates were isolated from normal chicken serum and were found to be mitogenic for mouse splenic lymphocytes as assayed by both blast transformation and [^{3}H]thymidine incorporation. The Fab' fragments of these carbohydrate-binding immunoglobulins were just as mitogenic as the divalent native antibody. Moreover, succinylated Fab' fragments, which probably would not form self-associating aggregates, showed similar mitogenic properties. All of these results indicate that, at least for saccharide-specific ligands, multipoint attachment and receptor cross-linkage on the cell to which the ligand is attached may not be a stringent requirement for activation.

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