Brief Definitive Report

GENERATION OF ANTI-TYPE III PNEUMOCOCCAL POLYSACCHARIDE HYBRIDOMAS FROM MICE WITH AN X-LINKED B-LYMPHOCYTE DEFECT

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CBA/N mice have an X-linked immunodeficiency that primarily influences the function of bone marrow-derived precursors of antibody-forming cells or B cells (1, 2). Animals bearing this defect fail to make a normal antibody response to a spectrum of thymus-independent (TI) antigens, TI-2; including the type III pneumococcal polysaccharide (SSS-III), but respond to TI-1 antigens (3). Although defective mice do not respond to phosphorycholine (PC) on TI-2-dependent or TI carriers, the activity of PC-specific helper T cells has been shown to be inhibited by anti-HOPC-8 idiotype serum, suggesting that V-region-gene expression in T cells is intact (4).

The serum antibody response to SSS-III is completely absent in defective mice; however, antigen-specific suppressor T-cells (Ts) can be generated by prior treatment with extremely low doses (5 ng) of SSS-III (5). This emphatically demonstrates the presence of V-region-gene products, capable of antigen-specific activation of either Ts or B cells in defective mice. To test whether B cells capable of anti-SSS-III responses exist in these mice, hybridomas were produced between plasmacytoma cells and spleen cells from immunized mice bearing the X-linked genetic defect to determine whether antibody synthesis and secretion could be enhanced.

Materials and Methods

Animals. 8-wk-old BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn.; 8-wk-old nonresponding (CBA/N × BALB/c male)F_1 (xid^+, CB mice) and responding (BALB/c × CBA/N male)F_1 (xid^-, BC mice) were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. Mice were bled from the retro-orbital plexus before killing and cell fusion for the measurement of serum antibody levels.

Immunizations. Mice were given an optimally immunogenic dose (0.5 μg) of SSS-III i.p.; 2 d later, they were given 300 μg concanavalin A (Con A) i.p. in saline (6). Mice were killed 5 d after immunization.

Cell Fusion. Hybridomas were produced as described by Margulies et al. (7) with slight modifications (K. Jin Kim and K. R. Schroer. Manuscript in preparation.). Pooled spleen-cell suspensions from four immunized mice were fused with polyethylene glycol to the NS1 plasmacytoma cell line of X63 (8). Fusion products were then cloned at a limiting dilution of 2×10^5 cultured spleen cells per well in 96-well plates (Costar, Data Packaging, Cambridge, Mass.). Culture supernates were assayed for antibody specific for SSS-III, at day 14, after fusion and subsequent culture.

Antibody Assays. Sera of immunized mice were tested for antibody specific for SSS-III by a modified Farr assay using 14% polyethylene glycol (mol wt, 6,000) to precipitate antibody-^{125}I-SSS-III complexes. Cell-culture supernates were assayed for antibody by radioimmunometric binding of ^{125}I-anti-Fab, (or iodinated anti-μ, anti-α, anti-γ1, or anti-γ2) to antigen-coated (SSS-III) polyvinyl chloride flexible microtiter plates (Cooke Engineering Co., Alexandria, Va.) (9). Binding in the radioimmunometric assays was >95% inhibitable with SSS-III.

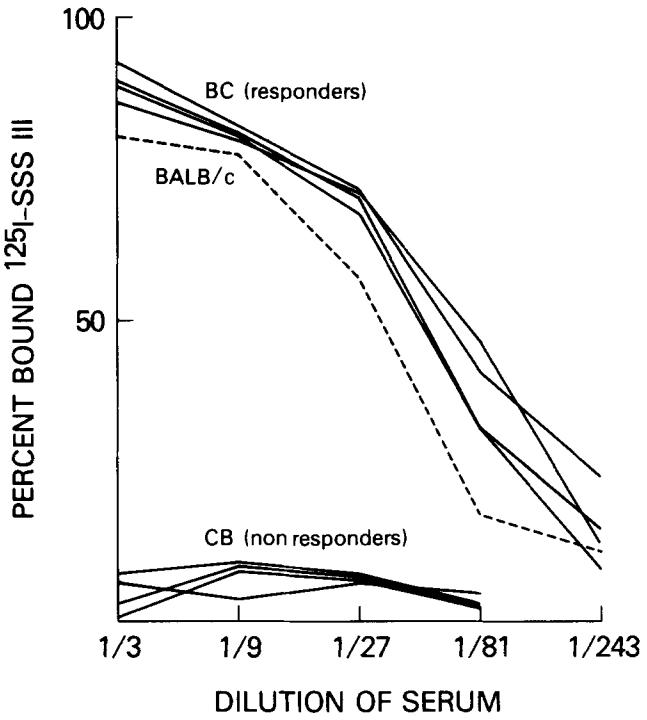

FIG. 1. Pre-fusion analysis of the mice used for generation of hybridomas by titration of serum antibody for binding to ^{125}I-SSS-III. Antigen-antibody complexes were precipitated by polyethylene glycol 6,000 at 14% final concentration. No detectable antibody is present in CB sera above background levels of nonspecific binding.

Results and Discussion

Serum antibody titers from immunized mice are compared in Fig. 1. Nonresponding CB mice bearing the X-linked genetic defect showed no detectable binding to SSS-III, whereas responding BC and BALB/c mice give rather typical binding curves (ABC 20:1/81–1/243). CB sera were shown by ^{125}I-anti-Fab binding to contain <20 ng/ml of anti-SSS-III antibody. Thus, significant amounts of antibody were not detectable in the serum of defective mice.

Spleen cells from hemizygous F_1 male mice were then fused to plasmacytoma cells after the mice were immunized with SSS-III and treated with Con A to optimize any expression of B-cell activity (6). The analysis of cell-culture supernatant fluids from cloned fusion products for binding to SSS-III was performed at 14 d. At that time, all clones were grown continuously in the presence of HAT (hypoxanthine, aminopterin, thymidine) to select for hybrids. This also ensures that the X chromosome derived from immunized spleen cells is present in the hybrid-cell heterokaryons because the X chromosome (HGPRT$^+$, xid$^+$) from the spleen-cells population provides protection against the lethal effects of HAT on parental myeloma cells, NS1 (HGPRT$^-$, xid$^-$). The analysis of positive and negative wells is shown in Table I for anti-μ specificity. Thus, 16 culture supernates gave binding comparable to levels obtained by specific dilutions of an affinity purified hybridoma standard (IgM, κ) that is maintained in

TABLE I

Radioimmunometric Analysis of Hybridoma Supernates

^{125}I-anti-μ binding*	Number of hybridoma wells		Equivalent quantity of IgM anti-SSS-III‡
	CB (nonresponder)	BC (responder)	
cpm			
<1,000	143	66	<3 ng/ml
1,000–5,000	4	1	—
5,000–10,000	1	0	100 ng/ml
10,000–15,000	1	3	—
15,000–20,000	0	0	1 μg/ml
20,000–25,000	2	0	—
25,000–35,000	0	4	10 μg/ml

* 0.010 ml of culture supernate from CB or BC mice hybridoma cultures were tested with 100,000 cpm ^{125}I-anti-μ for solid-phase binding to plastic plates.

‡ Affinity purified hybridoma (BALB/c, IgM, κ) anti-SSS-III (DB6-1).

our laboratory. Similar analyses were performed for other isotypes. The frequency and isotype distribution of the hybrids obtained is shown in Table II. Although the numbers are small and the frequency distributions are difficult to compare, a striking finding is the presence of SSS-III-specific hybrids from nonresponder, defective mice. In fact, the amount of antibody detected in culture fluids from such hybrids ranged from 10 ng/ml to 10 μg/ml. This occurred despite the fact that serum antibody can not be detected in the serum of such mice before cell fusion (Fig. 1).

The frequency of antigen-specific fusion products from nonimmunized mice is expected to be nearly zero. For another antigen, sheep erythrocyte (SRBC), it was thought highly unusual to obtain an antigen-specific frequency of 10% among hybrids making antibody specific for SRBC as described by Milstein et al. (8). Thus, they suggested that antigen-driven activation of B lymphocytes was the essential selective mechanism in generating a high fusion rate (10%). A 5% frequency using CB mice suggests that antigen-driven lymphocyte activation is indeed occurring, even if no specific evidence for such activation (the presence of serum antibody) is evident in vivo. This implies that the B cells of mice bearing the X-linked genetic defect possess antigen-specific receptors through which activation occurs. The reason why such activation is not expressed as antibody synthesis in mice bearing the X-linked genetic defect is not known, although there are several possibilities. If an intracellular mechanism for the assembly and/or export of immunoglobulin is defective, then the hybrid-partner cell (the NS1 plasma-cell tumor) may be reconstituting this defect. This would imply the existence of separate pathways for synthesis, assembly, and secretion of antibody when B cells are selected by TI-1 or TI-2 antigens. Immune suppressive regulation of secretion seems to be an unlikely possibility because others have clearly shown that B-cell responses may be transferred adoptively into defective mice (5, 10). Furthermore, speculation (11, 12) that V_H-gene expression is defective in conjunction with the inability to elaborate C_H genes commonly associated with polysaccharides (i.e., IgM and IgG3) seems unlikely because all eight isolated hybrids were IgM. The lack of hybrids of other classes should be interpreted cautiously because the number was small. (IgG3 assays were not performed in this investigation.) Whatever the mechanisms involved, the ability to generate hybrids from mice having

TABLE II

Frequency of Anti-SSS-III-specific Hybrids (Spleen × NSI Cells)*

Source of spleen cells	Total number of hybrids isolated	Number of clones with SSS-III-specific antibody,‡ class:				Number negative
		μ	α	γ1	γ2	
BALB/c	128	18	2	3	6	99
BC (responder)	79	8	4	1	0	66
CB (nonresponder)	151	8	0	0	0	143

* Spleen cells were removed at day 5 after SSS-III and Con A stimulation.

‡ Hybrids were examined for SSS-III-specific antibody secretion 14 d after fusion and plating at a limiting dilution.

a completely defective expressed repertoire to particular antigens suggests that hybridization must be used carefully to evaluate the expressed vs. the potential repertoire of antibody-forming cells.

Summary

(CBA/N × BALB/c male)F_1 mice bear on X-linked defect making them totally unresponsive to T-independent (TI), TI-2 antigens such as type III pneumococcal polysaccharide (SSS-III). We found that somatic cell hybrids between CB nonresponder spleen cells and NS1 plasmacytoma cells secreted antibody specific for SSS-III. The solid-phase binding of such antibody was completely inhibited by the addition of free antigen (SSS-III) and the amount of antibody detected in culture fluids ranged from 10 ng/ml to 10 μg/ml. Eight hybridoma clones were identified; all make antibody of the IgM class. These results indicate that the X-linked defect does not result in a deletion of a B-cell subset which responds to TI-2 antigens.

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