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Alloimmunization Induces an Autoantibody Reactive with a Common, Cryptic Antigen (41876)

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Abstract. C57BL/6 mice (haplotype H-2^b) were alloimmunized by a classical protocol using repeated injections of DBA/2 spleen and liver cells (H-2^d). The resulting polyclonal, hyperimmune antisera were highly cytotoxic to untreated and neuraminidase- (VCN)-treated DBA/2 spleen cells. An unexpected result was that the anti-H-2^d sera were also cytotoxic to VCN-treated C57BL/6 spleen cells. The cytotoxicity was limited to an IgM antibody and could be evoked by a single alloimmunization. The results demonstrate a new feature of the classical immune response, autoantibodies against a common, though cryptic murine antigen, designated Mo1.

Tissue grafts between murine strains of disparate haplotypes provokes both humoral and cellular immunity. Immune processing of allogeneic grafts probably involves the discrimination between self and non-self molecular domains of both the foreign tissue and host lymphoid cells. This recognition of self-antigens may lead to normal regulatory events, although an abberant response may generate autoreactive T and B cells (1). The induction of both autoantibody and autoreactive lymphocytes against self H-2 determinants has recently been reported (2, 3). I describe here the appearance of an autoantibody, detected by the lysis of neuraminidase-treated syngeneic cells, in the serum of C57BL/6 (H-2^b) mice following immunization to DBA/2 (H-2^d) strain tissue. This antibody is reactive with a cryptic, carbohydrate-containing antigen that is found on spleen cells from several mouse strains, and has been designated Mo1 for "common mouse antigen 1."

Materials and Methods. *Animals.* C57BL/6, DBA/2, B6D2F1, Balb/c, and SJL mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. CD1 mice were obtained from the Biomedical Research Center, Oral Roberts University.

Serum preparation. Normal C57BL/6 serum was obtained by bleeding 2- to 6-month-old female cagemates. The blood was collected on ice and pooled; the serum was isolated by centrifugation, aliquoted, and stored at −80°C. Hyperimmune, polyclonal C57BL/6 anti-DBA/2 serum was raised by a minimum of four ip injections of 3- to 6-month-old female C57BL/6 mice with 10^6 washed DBA/2 spleen and liver cells every 2 weeks. Four different batches of anti-DBA/2 serum were prepared in this manner and denoted anti-H-2^d serum. Preliminary studies showed that all anti-H-2^d serum produced >50% lysis of H-2^d-positive cells when tested at a dilution of 64-fold.

Serum fractionation. The hyperimmune serum was separated into two fractions by protein A–Sepharose CL4B chromatography, according to the method of Goding (4). The IgM-containing fraction (unbound and washed through the column by buffered saline, pH 8.2) and IgG fraction (eluted from the column by 1 *M* acetic acid in normal saline, pH 4.3) were reconstituted to original protein concentrations by positive-pressure dialysis. In addition, the hyperimmune serum was separated by Sephacryl S-300 gel filtration. One milliliter of serum was applied to a 1.5 × 116-cm column (flow rate, 13 ml/hr in PBS; room temperature) and the peaks corresponding to molecular weights of IgM (void volume) and IgG (150,000) were concentrated by positive-pressure dialysis back to the original serum volume. Double radial immunodiffusion of the peaks against class-specific antiserum (Cappel Laboratories, Cochranville, Pa.) showed that the void volume peak contained only IgM and the IgG peak contained IgG and some IgA immunoglobulin. These peaks were tested for cytotoxicity against untreated and neuraminidase-treated cells.

Cytotoxicity assay. The lysis of sham-treated and neuraminidase-treated cells was determined by a modified NIH technique, using trypan blue dye (2). Target spleen cells were washed twice in RPMI 1640 medium (containing 1% heat-inactivated fetal bovine serum, denoted 1% medium) followed by in-

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cubation in acetate buffer, pH 5.6, 37°C for 1 hr, with or without the presence of *Vibrio cholerae* neuraminidase (VCN) (Grand Island Biological Co., Grand Island, N.Y.) at 1 unit per 10^6 cells as previously described (8). Sham-treated cells were incubated in an equal volume of acetate buffer. The cells were then washed twice in 1% medium and 10^5 cells in 50 μl were added to 50 μl of serially diluted serum (or serum fractions), incubated for 45 min, 37°C followed by the addition of 5 μl of guinea pig serum (source of complement). All reactions required the presence of complement. After incubation of 1 hr, 25 μl of a 0.4% trypan blue solution was added to each well and a minimum of 200 cells were scored for viability. In the present study, our interest focused upon merely the presence or absence of significant cell lysis, due to the test sera compared to the cell lysis observed for cells incubated in the presence of complement alone. Hence, the percentage lysis is indicated in ranges of ten for simplicity of presentation. The lysis due to presence of complement alone was always less than 10%. χ^2 analysis of viable cell counts required that a minimum of 10–15% difference between samples be observed for significance at a level of $P < 0.05$. Intraassay variation between replicates was less than 8%. Maximum lysis of the VCN-treated C57BL/6 cells usually occurred at a twofold dilution of serum; hence all cell lysis was scored at this serum dilution.

Results. The data of Table I demonstrate the autoreactive phenomena. As expected, the sera of normal (nonimmunized) C57BL/6 did not possess cytolytic activity for either untreated or VCN-treated spleen cells obtained from C57BL/6, DBA/2, and B6D2F1 mice. Hyperimmune, polyclonal anti-DBA/2 sera (different batches) were cytotoxic to spleen cells from mice bearing the H-2^d haplotype (DBA/2; BALB/c, H-2^d; B6D2F1, H-$2^{b/d}$). However, these same sera were also cytotoxic to VCN-treated cells from C57BL/6; indeed, the sera lysed all VCN-treated spleen cells tested (SJL, H-2^s; CD1, outbred; C57BL/6, DBA/2, BALB/c, B6D2F1).

The cytolytic activity of these sera toward VCN-treated cells could be dissociated from the strong anti-H-2^d titer (usually greater than 1:64 for 50% cell death). The data of Figs. 1a–c demonstrate the removal of the anti-H-2^d activity of the hyperimmune sera after sequential absorption with 8, 12, and 16×10^8 DBA/2 spleen cells/ml of sera. However, in spite of this loss of alloreactive antibody, the absorbed sera retained the same level and titer of cytotoxicity against VCN-treated C57BL/6 cells (the use of the VCN-treated DBA/2 as target cells with absorbed sera is shown in Fig. 1d). This observation is consistent with the notion that the antigenic moiety of the VCN-treated H-2^b cell was not related to the H-2^d antigen(s). This idea was further strengthened by fractionating the antisera into IgM-containing and IgG fractions, using protein A–Sepharose chromatography. These fractions were tested for cytotoxicity against VCN-treated H-2^d cells (for clarity or comparison,

TABLE I. PRESENCE OF ANTI-C57BL/6 ANTIBODIES IN SERA OF ALLOIMMUNIZED C57BL/6 MICE

	Target cell lysis (%)							
C57BL/6 serum	C57BL/6	VCN-C57BL/6	DBA/2	VCN-DBA/2	B6D2F1	VCN-B6D2F1	CD1	VCN-CD1
Normal	<10	<10	<10	<10	<10	<10	<10	<10
Anti-DBA/2 (1)[a]	<10	>90	>80	>80	>80	>80	<10	>80
Anti-DBA/2 (2)	<10	>60	>80	>80	>80	>80	<10	>80

	VCN-C57BL/6	VCN-Balb/c	VCN-B6D2F1	SJL	VCN-SJL
Normal	<10	<10	<10	<10	<10
Anti-DBA/2 (1)	>80	>80	>80	<10	>80
Anti-DBA/2 (2)	>80	>80	>80	<10	>80
Anti-DBA/2 (3)	>80	>80	>80	<10	>80
Anti-DBA/2 (4)	>80	>80	>80	<10	>80

[a] Numbers denote different batch preparation of serum. Target cell lysis (%) was rounded to the nearest 10% for clarity of presentation.

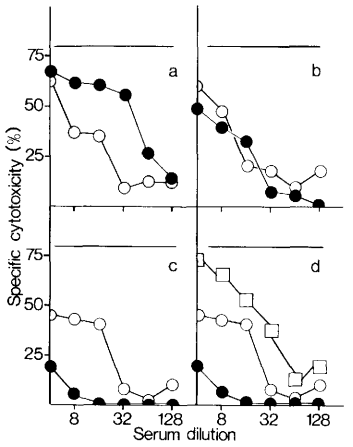

FIG. 1. The removal of the anti-H-2^d antibodies from the hyperimmune, polyclonal C57BL/6 anti-DBA/2 serum (a–c). ●, Cytotoxicity of anti-H-2^d serum toward untreated DBA/2 target cells after absorption with 8 (a), 12 (b), and 16 (c) × 10^8 DBA/2 cells/ml of serum. DBA/2 spleen cells were washed twice in normal saline and incubated in the above ratio for 2 hr, 4°C. The solid line indicates the lysis observed for an aliquot of unabsorbed serum. Cytotoxicity due to complement alone was less than 10%. ○, Cytotoxicity of absorbed serum toward VCN-C57BL/6 spleen cells. (d) □, Retention of cytotoxicity of absorbed serum toward VCN-DBA/2 spleen cells.

only the data for VCN-treated cells are shown) and the results are shown in Fig. 2. The specific cytotoxicity of the IgM fraction was somewhat lower than that of the unfractionated serum, evidently due to preparative procedures. The VCN-treated H-2^d cell was lysed by both the IgM and IgG antibody fractions of the anti-H-2^d sera. In contrast, only the IgM fraction was cytotoxic for the VCN-treated H-2^b cell, again suggesting the presence of autoantibodies having no specificity, per se, for the allogeneic cells. These results were confirmed by fractionation of the hyperimmune serum using Sephacryl S-300 gel filtration, and the results are shown in Table II. The IgM fraction was highly cytotoxic toward VCN-treated DBA/2, VCN-treated C57BL/6, and untreated DBA/2 cells, whereas the IgG fraction was cytotoxic for the allogeneic DBA/2 cells. Other studies (not shown) have determined that this IgM-mediated cytotoxicity can be blocked by D-galactosamine and D-glucosamine. The data of Table III show that a single injection of allogeneic cells was sufficient to induce detectable levels of the autoantibody in about 20 days.

Discussion. These data have shown that the allogeneic graft of H-2^d cells to the H-2^b recipient resulted in the induction of an autoantibody having specificity for a cryptic antigen, Mol, the expression of which was not limited to H-2^b cells. The generality of the phenomenon is currently being determined, e.g., the tissue distribution of Mol and the presence of the antigen among many inbred murine strains. The induction of the autoantibody is not associated with immune reactivity, per se, since high-titer C57BL/6 antisera to either sheep red blood cells of E1 strain measles virus did not contain autoreactive antibody (data not shown). The data are consistent with the notion that the lytic factor against VCN-treated C57BL/6 cells was IgM antibody since (a) cytotoxicity was complement dependent, (b) the cytotoxicity was abolished by treatment of the serum with 0.2 *M* mercaptoethanol, (c) the factor did not bind protein A, and (d) cytotoxicity eluted in the void volume of a Sephacryl S-300 column. The phenomenon described here is reminiscent of blood group reactivity (carbohydrate inhibition, IgM antibody), especially the T-antigen system reported by Springer and colleagues (5). They have described sialic acid-containing structures on a variety of normal and malignant tissue which serve as cryptic antigens of the M and N structures (T antigen) and most human sera contain naturally occurring antibodies to these normally hidden

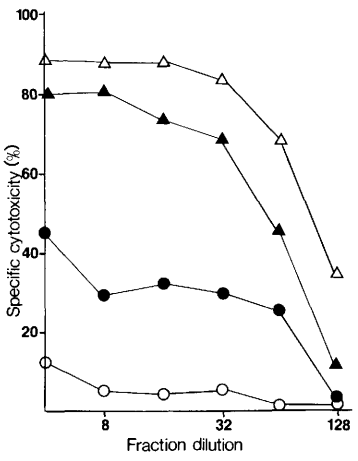

FIG. 2. Cytotoxicity of the IgG (open symbols) and IgM (closed symbols) fractions of hyperimmune, polyclonal C57BL/6 anti-DBA/2 serum toward VCN-treated DBA/2 (△, ▲) and VCN-treated C57BL/6 (○, ●) spleen cells.

TABLE II. FRACTIONATION OF C57BL/6 ANTI-DBA/2 SERUM BY SEPHACRYL S-300 GEL FILTRATION

	Target cell lysis (%)			
	C57BL/6	VCN-C57BL/6	DBA/2	VCN-DBA/2
Whole serum	<10	>80	>90	>90
IgM fraction	<10	>80	>80	>80
IgG fraction	<10	<10	>90	>90

Note. Values for percentage lysis represent nearest, lower whole number (in tens) for simplicity of presentation. Lysis due to complement alone was less than 10%. All values were from 1:4 dilutions of the indicated serum or fraction, although full-strength solutions and 1:2 and 1:8 dilutions gave the same results.

moieties. Indeed, the present results suggest that such antibodies may be a result of normal immune reactions.

Although autoantibodies are not uncommon in mice (6, 7), the autoantibody reported here has not been previously reported. At the present time, it is difficult to demonstrate the role of either this autoantibody or autoantigen, since the method of detection does not involve untreated lymphoid cells. The antibody may only react with a subpopulation of cells that express Mol in a noncryptic manner. There are two other circumstances that lead to the production of autoantibodies that have the properties described above (cytolytic to only VCN-treated cells, lack of H-2 specificity, presumably IgM class, carbohydrate inhibited): the genetically determined NZB autoimmune disease (unpublished observations) and the graft of the DBA/2 lymphoma L1210 into either the DBA/2 host or the B6D2F1 hybrid (8).

At the present time, the Mol autoantigen remains defined solely by serological methods. Efforts are currently directed to design experiments that demonstrate a functional (regulatory?) role for such autoantibodies and to biochemically characterize the Mol antigen.

TABLE III. PRODUCTION OF ANTI-C57BL/6 AND ANTI-DBA/2 ANTIBODIES WITH TIME FOLLOWING ALLOIMMUNIZATION OF C57BL/6 MICE

Donor C57BL/6		Target cell lysis (%)	
Injections	Bleed	VCN-BL/6	DBA/2
0	—	<10	<10
>4	—	>90	>90
1	Day 7	<10	>40
1	Day 14	<10	>50
1	Day 21	>80	<10
1	Day 42	>90	<10
2	Day 42	>70	>70

Note. Values for percentage lysis represent nearest, lower whole number (in tens) for simplicity of presentation. A group of C57BL/6 mice received ip injections of 10^6 DBA/2 spleen and liver cells; then separate groups of 3–5 mice were bled on Days 7, 14, 21, and 42. One group of five animals received a second ip injection of 10^6 DBA/2 cells on Day 14, and were bled on Day 42.

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1. Cohn M. Conversations with Niels Kaj Jerne on immune regulation: Associative versus network recognition. Cell Immunol **61**:425–433, 1981.
2. Risser R, Grunwald DJ. Production of anti-self H-2 antibodies by hybrid mice immune to a viral tumor. Nature (London) **289**:563–568, 1981.
3. Nakano K, Nakamura I, Cudkowicz G. Generation of F1 hybrid cytotoxic T lymphocytes specific for self H-2. Nature (London) **289**:559–562, 1981.
4. Goding JW. Conjugation of antibodies with fluorochromes: Modifications to the standard methods. J Immunol Methods **13**:215–226, 1976.
5. Springer GF, Desai R, Scanlon EF. Blood group MN precursors as human breast carcinoma-associated antigens and "naturally" occurring human cytotoxins against them. Cancer **37**:169–176, 1976.
6. Steele EJ, Cunningham AJ. High proportion of Ig-producing cells making autoantibody in normal mice. Nature (London) **274**:483–484, 1978.
7. Dresser DW. Most IgM-producing cells in the mouse secrete auto-antibodies (rheumatoid factor). Nature (London) **274**:484–486, 1978.
8. Killion JJ, Baker JR. Autoreactive factors identify tumor-host heterogeneity and responsiveness to immunotherapy. Cancer Immunol Immunother **12**:111–117, 1982.

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