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Isotype Profiles of Anti-Influenza Antibodies in Mice Bearing the *xid* Defect

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The humoral response to influenza A/PR8 virus was examined in the CBA/N and C_3J.*xid* strains of mice, both of which bear an X-linked genetic defect (*xid*), and in strains lacking this defect. Hemagglutination-inhibiting antibody titers and measurement of virus-specific antibodies by solid-phase radioimmunoassay indicated that the *xid* defect does not impair the production of an adequate anti-influenza antibody response. However, investigation of the isotypes of PR8 virus-specific antibodies disclosed a relative decrease in the levels of IgG3 and IgG1 in the *xid*-bearing strains. This was observed after both intraperitoneal immunization and aerosol infection. The isotype differences were not reflected in the susceptibility of these strains to influenza virus infection.

CBA/N mice bear an X-linked defect (*xid*) that interferes with the development of a B cell subset which displays the Lyb5 surface antigen (11). They do not respond to thymus-independent type 2 (TI-2) antigens such as trinitrophenylated (TNP)-Ficoll (6, 12). While anti-idiotype antibodies conjugated to Sepharose beads can stimulate proliferation of cells from these mice, soluble anti-immunoglobulin M (IgM) or anti-IgD will not (13). They also have low ratios of membrane IgD to membrane IgM (2).

C3H/HeJ mice possess a mutant gene (Lps^d), localized to chromosome 4 (15), which produces an unresponsiveness to both the immunological and pharmacological effects of endotoxin (16).

A C3H/HeJ congenic strain bearing the *xid* defect, C3J.*xid* (1, 4), has been developed. Interestingly, this strain bears all the defects of the parental CBA/N and C3H/HeJ strains in an enhanced form and also additional defects. Unlike cells of the parental strains, lymphocytes from these mice are unresponsive in vitro to thymus-independent type 1 (TI-1) antigens such as TNP-*Brucella abortus* (TNP-BA). Several B cell mitogens do not stimulate these cells, nor do anti-immunoglobulin antibodies conjugated to Sepharose beads. They are also unresponsive to certain T-cell-derived helper factors.

The antigen used in the present study was an influenza A virus, A/PR8/34 (H1N1) (PR8 virus). Previously, it had been reported that mice bearing the *xid* defect did not make a detectable hemagglutination-inhibiting (HI) antibody response after immunization with Formalin-inactivated influenza virus (3). In this communication, we provide evidence that mice bearing the *xid* defect develop adequate antibody responses to influenza virus antigens. However, significant differences in the relative amounts of different isotypes were observed in comparison to the responses of strains lacking this defect.

MATERIALS AND METHODS

Mice. C3H/HeJ and C3HeB/FeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine), and CBA/N mice were from Dominion Labs (Dublin, Va.). C3H/HeN mice were a gift from J. Mond (Uniformed Services University of the Health Sciences, Bethesda, Md.). C3H.*xid* mice were obtained from W. E. Paul (National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Viral antigen. The influenza virus, A/PR/8/34 (H1N1) (PR8 virus), was obtained from laboratory stocks, grown in the allantoic cavity of 10- to 11-day-old embryonated chicken eggs, and purified by previously reported methods (7).

Immunization. Five CBA/N, C3H/HeJ, and C3J.*xid* mice received an intraperitoneal injection of 10 μg of viral protein in 0.2 ml of saline on days 0 and 30. Orbital bleedings were done before the immunization on days 0 and 30 and also on days 5, 10, 30, 33, 40, 50, and 60. Pooled sera were employed in the solid-phase radioimmunoassay isotype determination (see below).

Infection. Five mice each from the CBA/N, C3H/HeJ, C3H/HeN, and C3HeB/FeJ strains underwent aerosol infection with a virus suspension containing 3×10^5 PFU/ml. Twenty-one days postinfection, mice were sacrificed and bled. Individual sera were used for the isotype determination.

HI antibody titration. Sera were treated with *Cholera vibrio* receptor-destroying enzyme before assay of HI titer. Tests were performed in microtiter plates, using 0.25% human type O erythrocytes and 3 hemagglutinating units of virus.

Isotype determination. The isotype content of PR8 virus-specific antibody in serum samples was determined by solid-phase radioimmunoassay. Microtiter wells were coated overnight with 50 μl of PR8 virus (50 μg/ml), washed three times with saline, and then coated for 1 h at 37°C with 1% bovine serum albumin in borate buffer (pH 8). After three washings with saline, 50 μl of serum or PR8 hemagglutinin-specific monoclonal antibodies of known isotype were added to wells and incubated for 1.5 h at 37°C. Appropriate dilutions of these monoclonal antibodies provided standard curves for each isotype (Fig. 1). IgM, IgG3, IgG1, IgG2b, and IgG2a PR8 hemagglutinin-specific monoclonal antibodies were available in our laboratory. The PR8 hemagglutinin-specific IgA monoclonal was a gift from Walter Gerhard (Wistar Institute, Philadelphia, Pa.). These standard monoclonal antibodies were purified by affinity chromatography on a PR8-Sepharose column. Their total protein content was then determined. After incubation, the plates were washed three times with saline, and 50 μl of ^{3}H-labeled goat anti-mouse isotype antibodies (a gift from P. Mongini, Hospital

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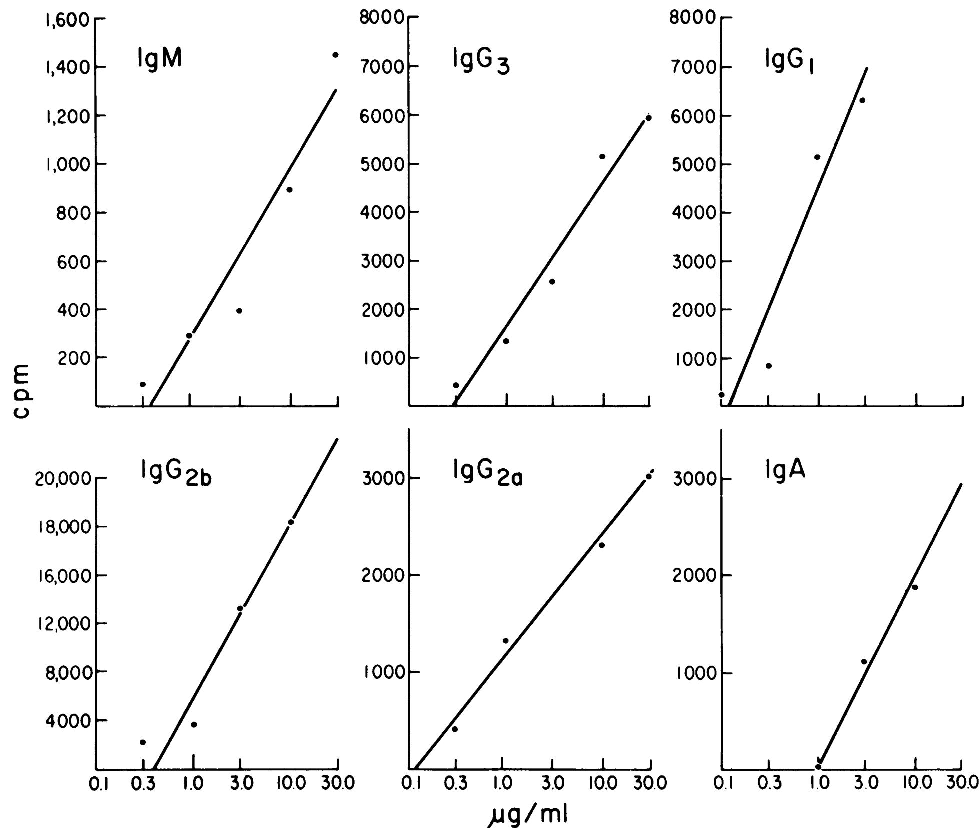

FIG. 1. Standard curves generated with affinity-purified, PR8 virus-specific monoclonal antibodies of known isotype. Each point represents the average of triplicate or quadruplicate determinations. Lines have been fitted by a linear regression analysis.

for Joint Diseases, New York, N.Y.) was added and incubated for 1.5 h at 37°C. The specificity of these reagents has been documented repeatedly in our laboratory (unpublished data) and in published reports from other laboratories (5, 14). After extensive washing, the radioactivity was counted in a scintillation counter.

PR8 virus-specific antibody of particular isotypes in the sera of mice after immunization was determined by using pools of sera of five mice in a similar fashion. Concentrations of isotype specific anti-PR8 antibodies were determined from the standard curves shown in Fig. 1. Concentrations of anti-PR8 antibodies of particular isotypes in the sera of mice after infection were obtained from individual sera (five mice per group). Background binding of sera from nonimmune mice was subtracted from all values.

RESULTS

PR8 virus-specific antibody response after intraperitoneal immunization. Mice from the CBA/N, C3H/HeJ, and C3J.*xid* strains received an intraperitoneal injection of 10 μg of viral protein on day 0 and were boosted on day 30. Serum samples were obtained by orbital bleeding on days 0, 5, 10, 20, 30, 33, 40, 50, and 60.

The kinetics of HI antibody production during primary and secondary responses in the different strains are shown in Fig. 2. No differences in the magnitude of response were observed.

Sera from five mice were pooled and employed in duplicate in the solid-phase radioimmunoassay for a determination of the isotypes of anti-PR8 virus antibodies (Fig. 3). The most significant differences among the strains were in production of the IgG1 and IgG3 subclasses of PR8 virus-specific antibodies. The IgG1 subclass was detectable only in the C3H/HeJ mice. The IgG3 production of this strain also exceeded that of the CBA/N and C3J.*xid* strains throughout both the primary and secondary response. The CBA/N secondary IgG3 response was somewhat greater than that of the C3J.*xid*. The IgM, IgG2b, and IgG2a responses were very similar in terms of kinetics and relative magnitudes in both the primary and secondary response. One exception occurred on day 20 of the primary response, when the IgM production by C3H/HeJ mice was significantly greater than that of the other strains.

Table 1 shows the ratios of gamma isotypes produced by the *xid*-bearing strains relative to the C3H/HeJ strain at two time points from both the primary and secondary responses. Most notably, the primary and secondary IgG3 and the secondary IgG1 responses were reduced in the *xid*-bearing strains. The ratios of IgG2b levels were somewhat decreased in the primary response, though levels in the secondary response were approximately the same. The ratios of IgG2a levels were variously increased or decreased at different time points.

PR8 virus-specific antibody response after aerosol infection. In a separate experiment, mice from the CBA/N, C3H/HeJ, C3H/HeN, and C3HeB/FeJ strains underwent aerosol infection. The mice were sacrificed 21 days postinfection, and isotype profiles of PR8 virus-specific antibodies were determined for individual serum specimens (Table 2). The IgG3 and IgG1 responses of the CBA/N strain were depressed relative to the three strains that do not bear the *xid* defect. These results are consistent with those of the intraperitoneal

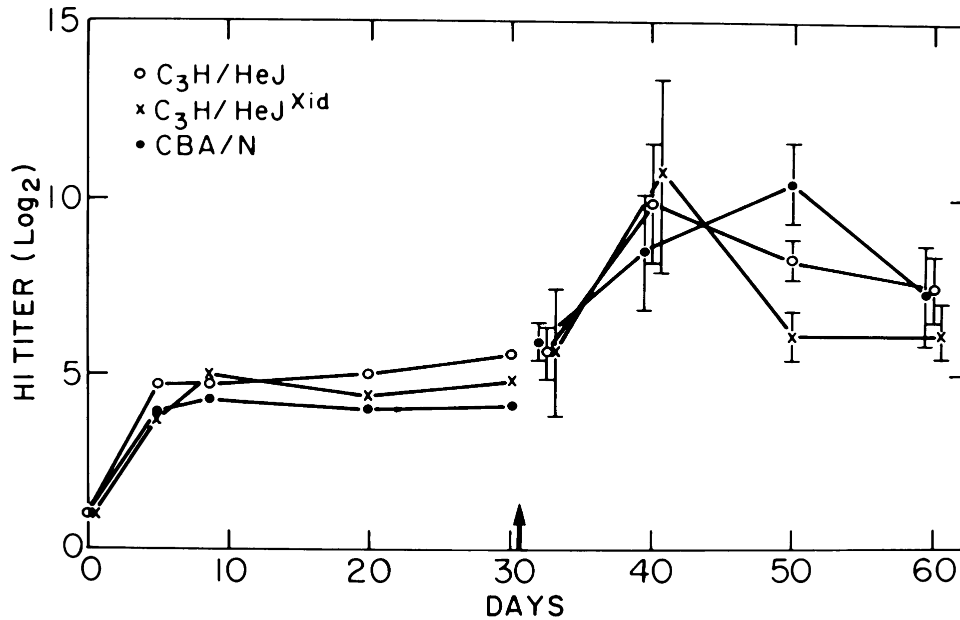

FIG. 2. HI titers (error bars are standard error of the mean) of individual serum specimens; five mice from each strain were immunized by intraperitoneal injection of viral protein on days 0 and 30. The mice underwent orbital bleeding at various times over a 60-day period. Arrow indicates secondary immunization on day 30.

immunization (Table 1). There were no consistent differences among the strains with regard to the IgM and IgG2a isotypes.

IgG2b levels in CBA/N mice were somewhat greater than those of normal mice, the reciprocal of the relationship with the IgG3 and IgG1 isotypes (Table 2). The IgG2b differences were viewed as less noteworthy because they were not confirmed by the results of the parenteral immunization (Table 1). IgA levels were increased in CBA/N mice relative to normal strains in this experiment.

DISCUSSION

It has been reported previously that HI antibody was not detectable in the sera of CBA/N mice 28 days after primary immunization with Formalin-inactivated virus. However, in the same study, it was noted that immunized CBA/N mice were resistant to lethal virus challenge (3). In contrast, in the present study, serum HI antibody titers at four time points in the primary response as well as the secondary response were indistinguishable from those of C3H/HeJ mice. We cannot explain the discrepancy between our results and those of Lucas et al. (3). However, since Formalin-inactivated virus does not elicit primary cytotoxic T cell responses in immunologically intact mice (10), the antiviral immunity noted in the Lucas et al. study and the intact HI antibody responses observed in CBA/N and C3J.*xid* mice in the present report are consistent with the hypothesis that the $Lyb5^+$ cell subset is not required for the production of virus-specific antibody of high avidity.

However, further investigation of the isotypes of PR8 virus-specific antibodies did disclose differences. The *xid* defect present in both the CBA/N and the C3J.*xid* strains is associated with decreased levels of IgG3 and IgG1 in both the primary and secondary responses relative to the levels of those isotypes in three strains that do not bear the *xid* defect (Tables 1 and 2). Most importantly, one of the normal strains, C3H/HeJ, is congenic to the C3J.*xid* strain (4). It should also be emphasized that in the case of the sera of aerosol-infected mice, reduced levels of IgG1 and IgG3 were observed in individual serum specimens, thus emphasizing the reproducibility of these results.

Though this decrease was seen after both parenteral immunization and aerosol infection, aerosol-infected mice had a greater reduction in IgG3 than in IgG1. The reciprocal relationship was found with parenterally immunized mice.

Given that the *xid* defect effects an inability to respond to certain carbohydrate antigens, one interpretation of the isotype pattern may be that the IgG3 and IgG1 levels are decreased because these subclasses are predominantly involved in responses to TI-2 carbohydrate antigens. However, comparison of results of solid-phase radioimmunoassays in which wells were coated with egg-grown virus, the immunogen in all these experiments, or with virus grown in Madin-Derby canine kidney cells, showed no differences (data not shown). This indicates that at best only a minor

TABLE 1. Ratios of gamma subclasses produced after parenteral immunization by $Lyb5^-$ strains (CBA/N and C3J · *xid*) relative to the $Lyb5^+$ strain (C3H/HeJ)

Strain	Days after infection[a]	Lyb5/$Lyb5^+$ ratio of IgG subclass: IgG3	IgG1	IgG2b	IgG2a
CBA/N:C3H/HeJ	10	0.13	—[b]	0.30	3.33
	20	0.16	—	0.47	8.33
	40	0.33	<0.06[c]	1.05	0.19
	50	0.47	<0.06	0.59	0.67
C3J · *xid*:C3H/HeJ	10	0.01	—	0.17	0.60
	20	0.21	—	0.40	2.40
	30	0.22	<0.06	0.67	1.94
	40	0.11	<0.06	1.25	0.52

[a] Mice underwent parenteral immunization on days 0 and 30. Ratios of subclasses produced by $Lyb5^-$ strains relative to the $Lyb5^+$ strain from two time points of the primary response (10 to 20 days) and two time points of the secondary response (40 and 50 days) are shown.

[b] —, No detectable response in any strain.

[c] Because there was no detectable response in the $Lyb5^-$ strains, the sensitivity of the assay (ca. 0.1 μg/ml) was divided by the $Lyb5^+$ value to arrive at the ratio.

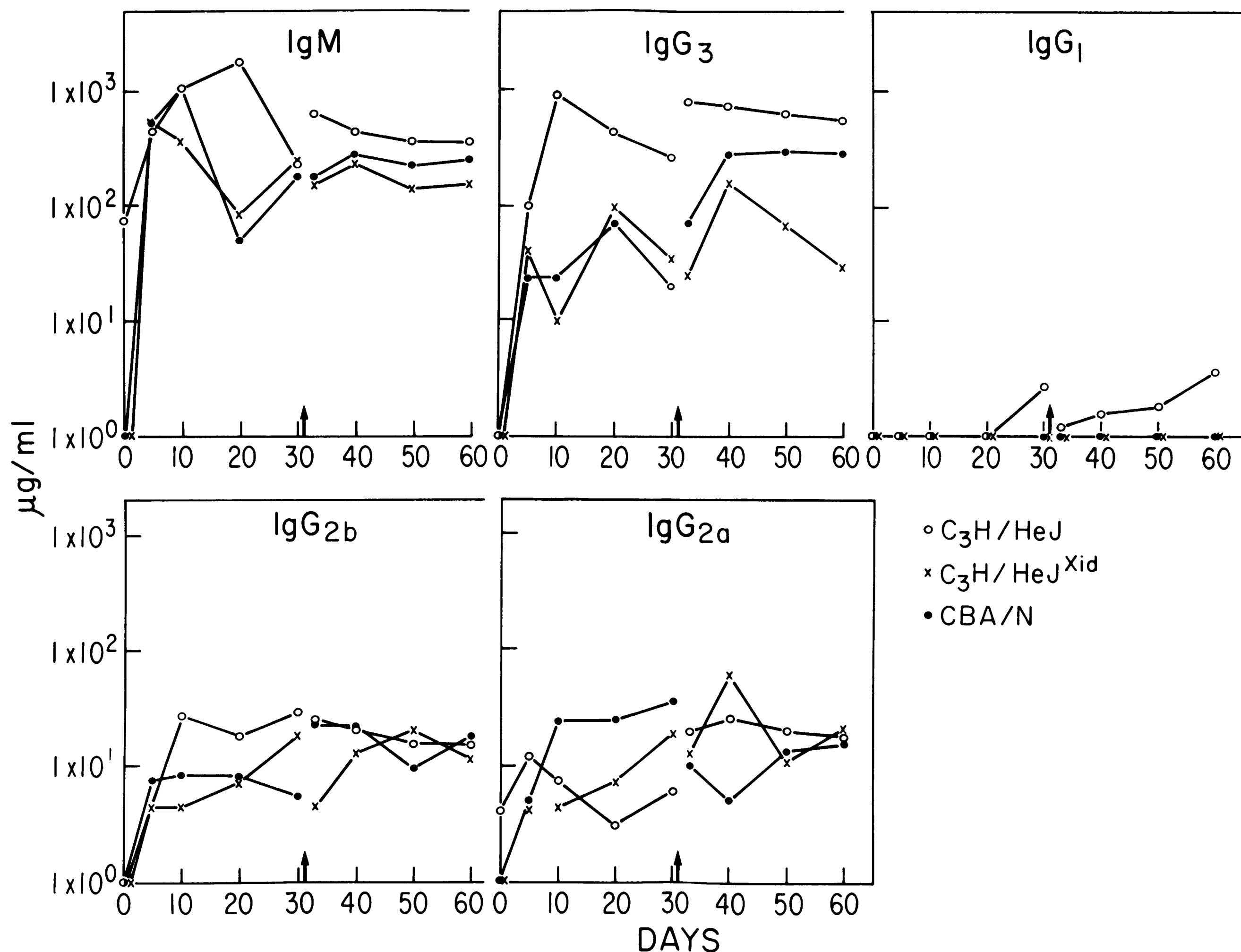

FIG. 3. Isotype profiles of PR8 virus-specific antibodies after primary and secondary parenteral immunization in C3H/HeJ, C3H/HeJ^{xid} = C3J.*xid*, and CBA/N mice. Serum specimens were employed in a solid-phase radioimmunoassay that utilized PR8 hemagglutinin-specific monoclonal antibodies of known isotype for development of standard curves and also ^{3}H-labeled isotype-specific antibodies. Arrow indicates secondary immunization on day 30.

component of the response was specific for host carbohydrate.

Two basic isotype patterns have emerged in the response of the *xid*-bearing strains to thymus-dependent antigens (9). A significant reduction of IgG3 with slight reductions in IgM and IgG1 has been seen in response to DNP-Hy (2,4-dinitrophenyl conjugated to *Limulus polyphemus* hemocyanin) (9). This is analogous to the response to TI-1 antigens and is most similar to our results with influenza virus. A second pattern, which is considered analogous to the TI-2 response, shows a reduction in all isotypes. This has been seen with (T,G)-A--L (9), sheep erythrocytes (8), and a thymus-dependent form of B512 dextran (5).

In the present experiments, differences in isotype production were not reflected in differences in susceptibility to influenza virus infection. In other experiments (data not

TABLE 2. Concentrations of PR8 virus-specific antibodies of different isotypes after aerosol infection[a]

Strain	Antibody concn (μg/ml) ± SEM					
	IgM	IgG3	IgG1	IgG2b	IgG2a	IgA
CBA/N	88 ± 49	2.4 ± 1	9 ± 2	83 ± 19	300 ± 155	29 ± 9
C3H/HeJ	103 ± 45	32 ± 9	17 ± 3	23 ± 2	544 ± 174	18 ± 6
C_3H/Hen	162 ± 91	33 ± 11	14 ± 4	56 ± 13	315 ± 117	2 ± 2
C3Heb/FeJ	29 ± 14	35 ± 8	24 ± 7	49 ± 13	120 ± 54	13 ±7

[a] Mice underwent aerosol infection with a virus suspension containing 3 × 10^5 PFU/ml. Twenty-one days postinfection, mice were sacrificed and bled, and individual sera were employed in the solid-phase radioimmunoassay isotype determination.

shown), it was observed that the CBA/N and C3J.*xid* strains did not differ from the nondefective strains in terms of 50% lethal dose, lung lesions, lung virus titers, or lung interferon production after aerosol infection. These results are consistent with those of Lucas et al. (3), who also observed no differences in mortality of CBA/N and Lyb5$^+$ CBA/CaJ mice after challenge.

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