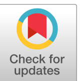
Proc. Natl. Acad. Sci. USA
Vol. 76, No. 9, pp. 4622–4626, September 1979
Immunology



Cell cytotoxicity due to specific influenza antibody production *in vitro* after recent influenza antigen stimulation

(hemagglutinin/radioimmunoprecipitation/interferon/vaccination)

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Communicated by Sidney P. Colowick, June 15, 1979

ABSTRACT Peripheral blood leukocytes, obtained from volunteers after vaccination or natural illness with influenza, were assayed for cytotoxicity against influenza virus-infected cells. Approximately 7 days after vaccination or the onset of respiratory illness, peak cytotoxicity was demonstrated in a chromium-release assay. Secretion of specific antibody against hemagglutinin from the leukocytes during *in vitro* incubation was demonstrated in quantities that would mediate the cell cytotoxicity observed. Antibody secretion was inhibited by exposure to cycloheximide but not by exposure to trypsin. The secretion of antibody against hemagglutinin from peripheral blood leukocytes occurred only at the time of maximal cytotoxicity. We thus demonstrate secretion of specific antibody *in vitro* after recent viral antigen stimulation. Moreover, this antibody is capable of conveying cytotoxic capacity to peripheral blood leukocytes that may be important in the recovery process from acute viral infection.

Virus-infected cells that express viral antigens on their surface can be lysed by different immunologic components and combinations of components. These include antibody and complement, T cells, antibody plus non-T cells, macrophages, and interferon plus natural killer cells (1–7). Studies in animals have demonstrated the importance of T cells in mediating early transient cytotoxicity after viral antigen stimulation (8–10). The few human studies that have been performed also demonstrate an early transient rise in cell cytotoxicity to virus-infected cells after antigen stimulation, but these studies have not delineated the effector mechanisms involved (11, 12).

Previous studies from this laboratory have demonstrated that human peripheral blood leukocytes (PBL) may exhibit cytotoxic activity toward influenza virus-infected cells (13–15). *In vitro* assays using PBL obtained from volunteers at various times after exposure to type A influenza antigens showed a sharp rise in cytotoxic activity on days 3–9 after infection or vaccination and a decline to lower stable levels by day 30 (14, 15). Cytotoxicity by PBL from volunteers who had a remote experience with influenza antigens was shown to be antibody dependent and mediated by an effector cell with characteristics of "K" cells (14). The experiments described in the present report are directed toward elucidating the mechanism(s) of the early transient rise in cellular cytotoxicity. The data indicate that PBL at this time are secreting specific antibody against hemagglutinin (AHAb) during a 4-hr period of *in vitro* incubation in quantities that are sufficient to mediate the cellular cytotoxicity observed.

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MATERIALS AND METHODS

Subjects. Seven volunteers participated in the studies. Volunteers 1 and 2 were tested sequentially after a natural respiratory illness proven for volunteer 1 to be caused by A/Texas/76 (H3N2) influenza virus (virus isolation and antibody rise) and, with similar testing, proven not to be caused by influenza virus for volunteer 2. Volunteers 2, 3, 4, 5, 6, and 7 were vaccinated with 400 chicken cell agglutinating (CCA) units of an investigational purified subunit vaccine prepared from A/Victoria/75 (H3N2) virus (kindly provided by R. Webster). At the time of vaccination, the six volunteers were healthy and gave no history of recent acute respiratory illness. Vaccination of volunteer 2 was 60 days after his previous noninfluenzal respiratory illness.

Cell Cytotoxicity. The method for cell cytotoxicity has been described (13–15). In brief, PBL were obtained from the buffy coat by Ficoll/Isopaque density centrifugation, washed twice in 50 ml of Eagle's minimum essential medium (16), and added to baby hamster kidney (BHK-21) cells, labeled with 0.1 mCi of ^{51}Cr ($Na_2{}^{51}CrO_4$; CJS-1, Amersham/Searle; 1 Ci = 3.7×10^{10} becquerels), that had been inoculated with the A/Port Chalmers recombinant influenza virus (H3pcNeq1) or with B/Hong Kong virus. Control target cells consisted of uninfected, chromium-labeled cells. After a 16- to 18-hr incubation, the monolayer cells were trypsinized, washed, and counted by trypan blue exclusion. Five $\times 10^4$ target cells in 0.1 ml were added to 2.5×10^6 PBL. After a 4-hr incubation at 37°C, 3 ml of cold Tris buffer containing 10% fetal calf serum was added, and each tube was centrifuged at $1000 \times g$ for 10 min. The radioactivity in the supernatant fluid and cell sediment from each tube was measured in a gamma counter (Amersham/Searle) to determine the percentage of chromium released from each tube.

The percentage of specific release was calculated from the following formula:

$$\text{Infected } [(L_v - S_v)/(M_v - S_v)] \\ - \text{uninfected } [(L_c - S_c)/(M_c - S_c)] \times 100,$$

in which L is leukocytes, S represents spontaneous release, and M represents maximum release after freeze-thawed lysis. The chromium release in the uninfected bottles ranged from −2.0 to 6.5%.

Preparation of Eluate and Assay of Antibody Release. For preparation of eluate, aliquots of PBL were incubated at 37°C for 30 min in serum-free minimum essential medium (10^6 lymphocytes per ml). After the incubation period, the PBL were

Abbreviations: PBL, peripheral blood leukocytes; AHAb, antibody against hemagglutinin; CCA units, chicken cell agglutinating units.

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centrifuged for 5 min at 800 × *g*. The eluate was removed and the cells were washed twice in minimal essential medium and recounted. These warmed and washed PBL were used directly in cytotoxicity assays as described above and samples were dispensed into conical centrifuge tubes (5 × 10^6 cells) with 1 ml of Tris-buffered saline/5% fetal calf serum and incubated at 37°C. At 0, 4, or 18 hr after the cells were dispensed, the tubes were centrifuged for 5 min at 800 × *g* and the supernatant fluid was removed and frozen at −70°C until assayed for antibody or added with heterologous PBL and tested for cytotoxicity. Trypan blue exclusion was performed on the cell pellet in each test.

In three vaccinated volunteers, PBL (5 × 10^6 cells) obtained on day 7 were treated with 2.5 μg of purified trypsin per ml (TPCK-treated, Worthington) for 30 min at 37°C and then washed twice with minimum essential medium/5% fetal calf serum. AT 0, 1, 4, and 18 hr after trypsin treatment, the tubes were centrifuged for 5 min at 800 × *g* and the supernatant fluid was removed and frozen at −70°C until assayed for antibody or added with heterologous PBL and tested for cytotoxicity. Similar studies were performed with day 7 PBL that had been incubated with 0.5–1 μg of cycloheximide per ml (Sigma) for 45 min at 37°C and then washed and reincubated with cycloheximide for the various incubation time periods.

Supernatants obtained from the PBL of volunteers 1–5 were added to warmed and washed PBL that had been obtained from an individual who had had no recent exposure to influenza virus antigen. These heterologous PBL gave low or no specific release. The percent specific release is derived from the percent release in the presence of prewarmed heterologous PBL and supernatant fluids less the percent release in the presence of PBL alone.

Adsorption of Supernatants. Eighteen-hour supernatants were adsorbed for 18 hr at 4°C with goat anti-human IgG 1:12 (γ chain specific). To facilitate precipitation, antibody-free carrier serum was added in equivalence with goat antiserum before adsorption.

Antibody Assays. AHAb binding capacity in both plasma and supernatant fluids was determined by radioimmunoprecipitation assays described earlier, except that data are here expressed as binding capacity rather than titer (17). All samples were tested with iodinated preparations of both A/Port Chalmers/73 and A/Victoria/75 hemagglutinin antigens. As an equally sensitive control test with a different antigen, samples were tested for antibody to the hexon subunit of adenovirus type 5 in a similar radioimmunoprecipitation assay (18).

Interferon Assay. A yield reduction assay using vesicular stomatitis virus as the challenge virus was used to measure inteferon activity in supernatant fluids (19).

RESULTS

Cell Cytoxicity during Natural Infection with Influenza. Volunteer 1 was tested for cell cytotoxicity after the onset of an acute respiratory illness caused by type A influenza virus (Fig. 1*A*). PBL and PBL that had been additionally warmed and washed had elevated levels of percent specific release (48.3 and 43.5%, respectively) to H3pcNeq1 (PC)-infected cells approximately 7 days after the onset of symptoms. By day 35, the percent specific release for each PBL fraction had dropped significantly (2.2 and 0%, respectively). Approximately 60 days after the onset of his illness, the value in the PBL fraction after warming and washing was 0%, although 24% was detected before the cells were warmed and washed.

Radioimmunoprecipitation assays of supernatant fluids obtained from warmed PBL before and after 4 and 18 hr of incubation revealed an increase in antigen-binding capacity for

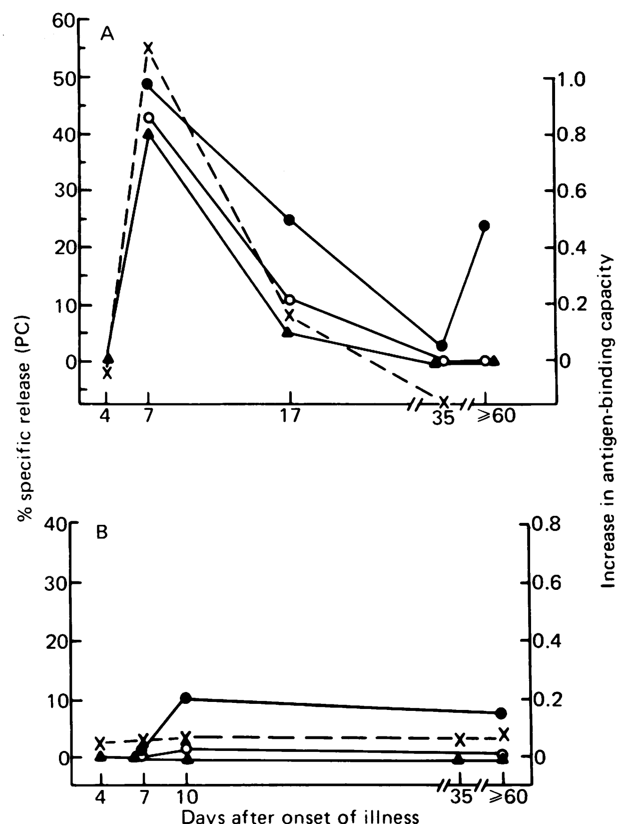

FIG. 1. Development of cell cytotoxicity and influenza AHAb by PBL after natural infection with A/Texas/1/76 (*A*) and after natural respiratory illness not caused by type A influenza (*B*). Cell cytotoxicity was measured by using PBL (●) and warmed and washed PBL (○). ▲, Increase in antigen-binding capacity (ng/50 μl) from the 0-hr to the 4-hr supernatants for each day tested. X, Cell cytotoxicity was measured by using heterologous PBL that had been warmed and washed and added to H3pcNeq1 (PC) infected target cells along with the 4-hr supernatants.

Port Chalmers hemagglutinin between the 0-hr and 4-hr supernatant samples only on days 7 and 17 after onset of illness (0.80 and 0.10 ng/50 μl, respectively). The 18-hr sample on day 7, but not on day 17, had an increase in antigen-binding capacity over the 0-hr sample that was greater than that measured for the 4-hr sample. No antibody activity was detected in the 0-, 4-, or 18-hr samples obtained on the other days tested (day 4, 35, or 60). All supernatants were tested and were negative for antibody to adenovirus type 5 hexon. The 4-hr and 18-hr supernatant samples obtained on day 7 were tested for interferon and all were negative (<1 unit/ml).

In order to evaluate the cytotoxic potential of *in vitro* released AHAb, we added the 4-hr supernatants obtained on each day with heterologous PBL that had been warmed and washed to H3pcNeq1- and B/Hong Kong-infected target cells. Only those supernatants obtained on days 7 and 17 conferred cytotoxic capacity to the heterologous PBL when they were added to the H3pcNeq1 target cells. No increases in cytotoxicity were demonstrated for any 4-hr supernatants added with heterologous PBL to the B/Hong Kong-infected cells.

Volunteer 2 was tested for cell cytotoxicity and specific influenza AHAb production after an acute respiratory illness that was not caused by type A influenza virus (Fig. 1*B*). No significant changes in cell cytotoxicity were found in the days tested. In addition, supernatants obtained from the PBL that were warmed, washed, and subsequently incubated failed to dem-

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onstrate AHAb activity on any of the days tested. Moreover, no increase in cytotoxicity was demonstrated when these supernatants were added with heterologous PBL to H3pcNeq1- or B/Hong Kong-infected cells.

Cell Cytotoxicity after Vaccination with Inactivated Influenza Virus Vaccine. Volunteer 2, who had had a noninfluenzal viral illness over 60 days earlier, and two other healthy volunteers (volunteers 3 and 4) were vaccinated with a purified subunit vaccine prepared from A/Victoria/75 (H3N2) virus. Cell cytotoxicity and AHAb presence in supernatants were measured before and at various times after vaccination. All three volunteers had low but detectable AHAb to A/Port Chalmers/73 and A/Victoria/75 prior to vaccination and developed significant plasma antibody rises to the A/Port Chalmers and A/Victoria hemagglutinin by day 35.

On day 7 after vaccination, all three vaccinees had elevated percent specific release with either PBL or the PBL that had been warmed and washed (Table 1). Cytotoxicity was markedly reduced in PBL that had been warmed and washed on all other test days after vaccination.

An increase in antigen-binding capacity for A/Port Chalmers hemagglutinin was detected in the 4-hr supernatants on day 7 in all three vaccinees (Table 2). Small increases in antigen-binding capacity were also seen on day 20 in volunteer 2 and on day 35 in volunteer 3. However, 18-hr supernatants on day 20 in these volunteers did not increase further when compared with the 4-hr samples. All supernatants on all days were negative for antibody to adenovirus type 5 hexon by radioimmunoprecipitation, and the 18-hr supernatants on day 7 of volunteers 2 and 3 had no detectable interferon (<1 unit/ml).

The 4-hr supernatants (0.5 ml per tube) on day 7 of volunteers 2 and 3 and the 18-hr supernatants of volunteer 4 conferred cytotoxic capacity to the H3pcNeq1-infected target cells when added with heterologous PBL that had been warmed and washed (Table 2). The low level of cytotoxicity in volunteer 2 may be attributable to the low percent of infected target cells used in that particular experiment (immunofluorescent-positive cells ≤40%). No increases in cytotoxicity were seen for the 4-hr supernatants obtained on other days when compared with the cytotoxicity of the PBL alone, and no increase in cytotoxicity was seen for any of the 4-hr supernatants added with heterologous PBL to B/Hong Kong-infected target cells.

Table 1. Cell cytotoxicity after vaccination with inactivated influenza virus vaccine*

Volunteer	Day after vaccination	% specific release†	
		PBL‡	Incubated PBL‡
2	0	7.7	0.4
	7	28.2	23.7
	20	12.0	3.9
	35	ND	ND
3	0	18.6	0.6
	3	8.1	3.6
	7	71.3	64.5
	35	ND	ND
	60	5.6	−0.5
4	0	ND	ND
	3	15.0	2.5
	7	35.0	20.0
	35	ND	ND
	60	11.5	4.6

* Volunteers were vaccinated with 400 CCA units of A/Victoria/75 influenza virus vaccine.

† Percent specific release to H3pcNeq1-infected BHK-21 cells; % chromium release from uninfected cells ranged from −2.0 to 6.5%. ND, not done.

‡ PBL were added to target cells in a ratio of 50:1 after they were washed twice or after additional incubation of 37°C for 30 min and two further washes.

Table 2. Release of influenza antibody from PBL after vaccination with inactivated influenza virus vaccine*

Volunteer	Day after vaccination	Plasma AHAb†	Increase in Ag-binding capacity†	% specific release‡
2	0	25	0.00	4.3
	7		3.44	7.3
	20		0.15	4.3
	35	1575	0.00	ND
3	0	60	0.00	−2.6
	3		0.00	−2.4
	7		0.16	26.2
	35		0.02	4.2
	60	905	0.01	ND
4	0	60	0.00	−2.8
	3		0.00	−3.8
	7		0.03 (0.18)§	5.4
	35		0.00	−6.1
	60	905	0.00	ND

* Volunteers were vaccinated with 400 CCA units of A/Victoria/75 influenza virus vaccine.

† Antigen-binding capacity (in ng/50 μl) was determined by radioimmunoprecipitation with hemagglutinin of A/Port Chalmers/1/73.

‡ Heterologous lymphocytes were incubated at 37°C for 30 min, washed twice, and added to target cells with 0.5 ml of the 4-hr supernatants obtained after vaccination. Eighteen-hour supernatants were used for volunteer 4. ND, not done.

§ Value in parentheses, at 18 hr.

Effect of Trypsin and Cycloheximide on Release of AHAb from PBL. Volunteers 5, 6, and 7 were vaccinated with the same inactivated vaccine and their PBL were removed and tested for release of AHAb on day 7. An aliquot of PBL was incubated in minimum essential medium/5% fetal calf serum after they had been warmed and washed, and supernatants were removed 0, 1, 4, and 18 hr later and assayed for antigen-binding capacity by radioimmunoprecipitation. A second aliquot of PBL was incubated with trypsin or cycloheximide, and supernatants were removed as before and tested in a similar manner. Trypsin treatment did not alter the production of AHAb compared with the PBL incubated only with medium (Table 3). However, cycloheximide significantly inhibited the AHAb released from PBL. Viability of PBL ranged from 65 to 90% at 18 hr.

Sufficient supernatant fluids were available from volunteer 5 for testing in a cytotoxicity assay. Supernatant fluids from the warmed and washed and the trypsin-treated PBL, but not the cycloheximide-treated PBL, conferred cytotoxicity when added to heterologous PBL.

Effect of Supernatant Adsorption with Goat Anti-Human IgG. Eighteen-hour supernatants obtained on day 7 after illness in volunteer 1 or on day 7 after vaccination in volunteers 2 and 3 were tested for the ability to confer cytotoxic capacity after adsorption with goat anti-human IgG. The adsorbed supernatant samples had no detectable AHAb activity as measured by radioimmunoprecipitation and failed to confer cytotoxic capacity to H3pcNeq1-infected cells when added with heterologous PBL that had been warmed and washed.

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Table 3. Release of influenza antibody from PBL after vaccination with inactivated influenza virus vaccine*

Volunteer	Treatment of PBL†	Increase in Ag-binding capacity‡		
		1 hr	4 hr	18 hr
5	Medium	0.07	0.19	0.17
	Trypsin	0.03	0.18	0.24
	Cycloheximide	0.01	0.03	0.06
6	Medium	0.02	0.05	0.15
	Trypsin	0.02	0.05	0.14
	Cycloheximide	0.00	0.00	0.00
7	Medium	0.00	0.04	0.10
	Trypsin	0.05	0.17	0.29
	Cycloheximide	0.00	0.00	0.00

* Volunteers were vaccinated with 400 CCA units of A/Victoria/75 influenza virus vaccine.

† Tests were performed on PBL obtained on day 7 after vaccination. Medium, minimum essential medium/5% fetal calf serum. Trypsin, 2.5 μg/ml for 30 min at 37°C; cycloheximide, 0.5–1.0 μg/ml.

‡ Antigen-binding capacity (in ng/50 μl) was determined by radioimmunoprecipitation with hemagglutinin of A/Victoria/75.

DISCUSSION

These experiments indicate that PBL obtained after natural illness or vaccination with type A influenza viruses are actively secreting AHAb 7 days after antigen stimulation. The AHAb that is secreted from PBL during a 4-hr incubation is capable of conferring cytotoxic capacity to heterologous PBL when added to target cells infected with influenza A but not those infected with influenza B virus. Trypsin treatment of PBL did not alter the secretion of AHAb whereas cycloheximide, an inhibitor of protein synthesis, significantly reduced the measurable AHAb during an 18-hr incubation. The failure of trypsin to inhibit the reaction suggests that the antibody is not cytophilic. The failure to detect interferon secretion demonstrates that one is not observing interferon-mediated natural killer cell reaction (6). Therefore, the early transient rises in cell cytotoxicity by PBL that persisted after incubation for 30 min at 37°C and repeat washing in our earlier studies was probably due to the AHAb secreted in the 4-hr interval used for the chromium release assay.

The demonstration in humans of secretion of specific antibody *in vitro* after recent *in vivo* viral antigen stimulation has not been reported previously. Similar results in mice with nonviral antigens have been reported and are consistent with our results. Schirrmacher *et al.* (20) demonstrated that antigen-coated chicken erythrocytes were lysed in the presence of nonimmune spleen cells and an active factor that was released from immune spleen cells. The immunoglobulin nature of this factor was indicated by its precipitability in half-saturated ammonium sulfate and its removal by matrix-bound anti-mouse gamma globulin. Recent studies have confirmed these findings, with sheep erythrocytes as the immunizing antigen (21).

The extent of influenza A antibody synthesis by PBL remains to be determined. Because these volunteers had been previously exposed to influenza A virus, synthesis of AHAb to other H3 variants, or to earlier subtypes, may also have occurred. AHAb to both A/Port Chalmers and A/Victoria hemagglutinin was detected in the supernatants on day 7 after vaccination, but only by radioimmunoprecipitation. Other standard tests for influenza antibody (i.e., neutralization or hemagglutination inhibition) would not have detected the low levels of antibody in these supernatant fluids.

There was an apparent lack of correlation between the percent specific release measured in PBL, the antibody levels detected in the supernatants, and the percent specific release measured when the supernatants were added to heterologous PBL. The 4-hr supernatants obtained on day 7 gave lower percent specific release when added to heterologous PBL than when day-7 PBL were tested prospectively. These differences in cytotoxicity levels are due, in part, to the smaller amount of supernatant fluid (0.5 ml/tube or half of the remaining fluid) that was used in the experiments with the heterologous PBL. In addition, the radioimmunoprecipitation assay may measure subclasses of IgG that have varying cytotoxic potential. The vaccine used in the present study is composed of only the surface glycoproteins, the hemagglutinin, and neuraminidase. Therefore, antibodies directed against the internal viral proteins should not have been synthesized and could not participate in the cytotoxicity reaction.

Our earlier studies had implicated a nonphagocytic, nonadherent, Fc-bearing lymphocyte, presumably a K cell, in mediating cell cytotoxicity to influenza A-infected target cells remotely after antigenic exposure (14). However, B cells (22, 23), K cells (24), macrophages (25), and even T cells (26, 27) have also been implicated as effector cells in antibody-dependent cell cytotoxicity. K cells may also be the cell mediating the early transient cytotoxicity, but the specific cells involved have not yet been delineated. It may be that the cell releasing the antibody also is the effector cell in the cytotoxic reaction.

Because all seven subjects in the present study had experienced infection with some related type A virus HA in the past, their cytotoxicity response and synthesis of influenza virus AHAb are manifestations of a secondary antibody response. The changes and mechanisms of cytotoxicity that may accompany a primary immune response are yet to be defined. Nevertheless, in the present study, the early cytotoxicity responses after virus infection appear to be maximal during the period of recovery from infection and illness, thereby suggesting the possibility of a role for this immune response in the recovery process.

We thank Bonnie Hughes, Barbara Baxter, and William Hollis for their excellent technical assistance. This work was supported by Contract NO1-AI-42528 from the National Institute of Allergy and Infectious Diseases.

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