JOURNAL OF VIROLOGY, Dec. 1984, p. 839–845
0022-538X/84/120839-07$02.00/0

Vol. 52, No. 3

In Vitro Enhancement of Human Natural Cell-Mediated Cytotoxicity by Purified Influenza Virus Glycoproteins

D. JIT S. ARORA,[1]* MICHEL HOUDE,[1] DOMINIC M. JUSTEWICZ,[1,2] AND ROSEMONDE MANDEVILLE[2]

Virology[1] and Immunology[2] Research Centers, Institut Armand-Frappier, University of Quebec, Laval, Quebec H7N 4Z3, Canada

Received 16 May 1984/Accepted 20 August 1984

The role of the glycoproteins of influenza virus, hemagglutinin (HA), and neuraminidase (NA) in the in vitro stimulation of natural cell-mediated cytotoxicity (NCMC) or natural killer activity of human peripheral blood lymphocytes was evaluated with radiolabeled K562 cells as target cells in an overnight chromium release assay. Three different approaches were used. (i) Purified viral proteins were obtained by extraction with Nonidet P-40, separation on a sucrose gradient, and further purification by affinity chromatography. Ficoll-Hypaque-purified peripheral blood lymphocytes exposed to HA or NA individually or to a mixture of both significantly increased NCMC (32 to 50%). (ii) Treatment of HA and NA with their respective homologous antisera or $F(ab')_2$ antibody abrogated the stimulation of NCMC by these glycoproteins. (iii) Virions treated with proteolytic enzymes resulted in viral cores lacking either HA or NA or both activities. Compared to whole virions, viral cores devoid of HA activity only induced a 50% increase in NCMC, whereas viral cores lacking HA activity and with traces of NA activity stimulated only 10% of the NCMC. These results suggest that influenza virus-induced cell-mediated cytotoxicity is largely due to its glycoproteins.

Specific and nonspecific immune responses have been shown to play an important role in the recovery from influenza virus infection (reviewed in references 10, 13 through 15, and 26). Thus, specific immune responses, such as local antibody secretion in the respiratory tract, have an important role in neutralizing some or all of the challenge virus (10, 26). Influenza virus infection also induces nonspecific responses, such as the stimulation of natural cell-mediated cytotoxicity (NCMC) and the production of interferon (13, 14).

Recently, considerable attention has been focused on the induction or enhancement of NCMC because of its potential to halt or reduce early virus replication (4, 7, 8, 15, 17, 37). Natural killer (NK) activity increases in humans shortly after influenza virus infection; results from a clinical assay showed a two- or threefold increase in NK activity within the first 3 days in volunteers who had received live influenza virus H1N1 intranasally (15). Moreover, this increased NK activity could be correlated with the early signs of recovery (15). NK effector cells may have an in vivo role in the prevention or limitation (or both) of spread of virus infections, particularly in the early stages of infection, and at the sites of initial virus replication, until specific cytotoxic T lymphocytes and antiviral antibodies are generated (13, 18, 30). It was also suggested that failure of this early defense mechanism might favor the virulence over the host's immune system later in infection (7, 13).

Studies with different viruses have also shown that, in vitro, whole virions as well as specific viral proteins can induce or enhance NCMC (1, 4, 7, 19). For example, purified hemagglutinin (HA) and fusion glycoproteins of measles virus can induce cell-mediated cytotoxicity (CMC) (7). Although the mechanism by which virions or their components stimulate NK activity is still controversial, two mechanisms have been suggested. Earlier studies (11, 18, 27, 31–33, 37) demonstrated that NK activity is proportional to or dependent on the interferon (IFN) produced, either by the target cell or the NK effector cell. Furthermore, IFN was shown to be the major internal mediator of NK activity (4, 11, 27, 33, 37). More recent studies, however, have demonstrated that the activation of NK activity is IFN independent (4, 6–9, 16–18, 37, 39). It is, nevertheless, clear that IFN-dependent and IFN-independent CMC are two discrete ways of activation and expression of virus-induced CMC.

With regard to influenza virus, the viral components involved in the stimulation of NCMC are little known; we have recently shown that an HA1-specific monoclonal antibody did inhibit virus-induced CMC (19a). In the present study, a more direct approach was conceived to demonstrate clearly the role of the two major glycoproteins, HA and neuraminidase (NA), in the in vitro stimulation of NK cell activity of human peripheral blood lymphocytes (PBL).

MATERIALS AND METHODS

Virus purification. Influenza virus A/Port Chalmers/1/73 (H3N2) (A/PC) was propagated in the allantoic cavity of 11-day-old embryonated eggs and incubated at 35°C for 48 h. Allantoic fluid was harvested under sterile conditions and stored at −80°C until used. Virus-infected allantoic fluid was filtered through glass wool, and chicken erythrocytes were added to the filtrate. Virus particles that were adsorbed to and eluted from erythrocytes in the presence of a receptor-destroying enzyme (21) were further purified by simplified sucrose density gradient centrifugation (3). These purified virions form a transparent pellet that can be readily suspended in 0.85% NaCl containing 0.08% sodium azide, giving a white, intensely opalescent suspension from which nothing is deposited by low-speed centrifugation.

Isolation and purification of HA and NA. Purified virus was treated with Nonidet P-40. The suspension was centrifuged, and the supernatant was layered on a preformed sucrose density gradient (5 to 20%). Fractions containing the HA and NA activities were collected, and these proteins were further purified by affinity chromatography with appropriate adsorbents as already described (2).

* Corresponding author.

IFN. Human leucocyte IFN-α was obtained from the Research Resources Branch of the National Institute of Health. A concentration of 50 IU/ml was used.

Protein determination. Protein concentration was determined by the Lowry et al. technique (22).

Determination of HA activity. Samples were serially diluted twofold with phosphate buffered saline (0.01 M sodium phosphate buffer [pH 7.2] containing 0.85% NaCl) and added to a washed chicken erythrocyte suspension (0.5%) as already described (2).

Determination of NA activity. The NA activity was measured by incubating samples at 37°C with fetuin (25 mg/ml) for 18 h. Liberated N-acetylneuraminic acid was determined by absorbance at 549 nm (2).

Treatment of virions with proteolytic enzymes. The proteolytic enzymes (pronase, chymotrypsin, and bromelain) were used at the following final concentrations: pronase, grade B (Calbiochem, Los Angeles, Calif.), at 1.05 μg/ml (28); chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) at 0.024 μg/ml (27); and bromelain, grade II (Sigma), at 15 μg/ml (5, 24). All enzymes were diluted with phosphate-buffered saline. Treatment of purified influenza virions (10 μg/ml) was carried out at 37°C for 60 min. This was followed by centrifugation for 1 h at 90,000 × *g*. The sedimented viral cores were suspended in 200 μl of phosphate-buffered saline and tested for their protein content, HA and NA activities, and for their capacity to induce NCMC.

Preparation of human lymphocytes. PBL from normal healthy male donors (20 to 45 years of age) were treated with carbonyl iron to remove monocytes and macrophages and then layered on a Ficoll-Hypaque density gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). Washed PBL were adjusted to a concentration of 4×10^6 viable cells per ml (as assessed by trypan blue exclusion) with RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 4 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and antibiotics as described previously (23).

Target cells. K562 cells, used as target cells, were labeled with $Na_2{}^{51}CrO_4$ (specific activity, 2 mCi/mg; Frosst, Kirkland, Quebec, Canada) for 45 min at 37°C, washed twice, and adjusted to a concentration of 10^5 viable cells per ml.

Cytotoxicity assay. NK activity was measured by an overnight ^{51}Cr release assay as described previously (23). Briefly, variable numbers of lymphocytes and chromium-labeled K562 cells were placed in round-bottom microtiter plates (Linbro, New Haven, Conn.) to give effector/target cell (E/T) ratios of 20:1, 10:1, 5:1, and 2.5:1. After centrifugation for 2 min at 200 × *g*, the microplates were incubated for 16 to 18 h at 37°C (except in 4-h, short-term experiments) and centrifuged for 10 min at 200 × *g*, and the supernatant was collected by the Titertek Harvesting System (Flow Laboratories, McLean, Va.). The percent cytotoxicity (referred to as NK activity) was calculated as follows. % specific ^{51}Cr release = [cpm (test) − cpm (spontaneous)]/[cpm (maximum) − cpm (spontaneous)] × 100 where cpm is counts per minute. All experiments were done in triplicate, and the results presented are the means of three independent observations (± standard deviations). The cpm (spontaneous) was determined by counting the radioactivity of the supernatant of the centrifuged labeled target cell suspensions, whereas the cpm (maximum) was the radioactivity of resuspended labeled target cells.

Virus-induced CMC. Samples (50 μl) of intact virus or viral cores or purified glycoprotein (NA, HA, or a mixture of both) were added directly to the test system at the E/T ratio of 10:1. A series of 10-fold dilutions of each preparation was used in different experiments starting at 3×10^{-1} or 11×10^{-1} μg of protein per ml, depending on whether preparations of intact virus or viral cores or purified glycoprotein were used.

Preparation, characterization, and use of monospecific antisera and F(ab')$_2$ fragments. New Zealand white rabbits of 7 to 11 kg were immunized with either intact virions (A/PC) or with fractions containing purified glycoproteins (HA and NA). Emulsions were prepared by dilutions in equal volume of incomplete Freund adjuvant. The quantities injected varied from 40 to 150 μg of protein per injection per rabbit. The first two injections were given intramuscularly at an interval of 1 week, whereas the third injection was given subcutaneously 40 days after the second injection. Rabbits were bled 12 days after the last injection. The specificity of the antisera was evaluated by double immunodiffusion and inhibition tests (2). It is important to point out that the monospecific anti-HA antiserum neither neutralized the NA activity nor gave a precipitin line by double immunodiffusion test against NA, whereas the antiserum produced by injection of purified viral HA neutralized its HA activity, gave precipitin lines when double immunodiffusion tests were performed, and did not cross-react against viral NA.

The separation of globulins from the serum was achieved by adding 0.5 volume of saturated ammonium sulfate to 1 volume of serum. After centrifugation at 4,000 × *g* for 15 min, the supernatant was discarded, and the precipitate was washed with 40% ammonium sulfate and suspended in water. The divalent antibody fragments F(ab')$_2$ were obtained by pepsin digestion. The procedure followed was essentially that of Nisonoff et al. (25) with some modifications. The immunoglobulin precipitate of the antiserum was dialyzed against 0.1 M acetate buffer (pH 4.3), and crystalline pepsin at a globulin/pepsin ratio of 50:1 was added to the solution. The mixture was incubated in a 37°C water bath for 8 to 14 h and then cooled in an ice bath. The pH was adjusted to 8.0. Further purification of F(ab')$_2$ was achieved by gel filtration on Sephacryl S-200 or S-300 (Pharmacia Fine Chemicals). The purity of F(ab')$_2$ was checked by agar gel immunodiffusion with the appropriate antiserum.

Inhibition of virus-induced CMC by monospecific antisera. The effect of monospecific antisera on the virus-induced CMC was determined by the following two types of assays: (i) the antisera were preincubated with the antigen suspen-

TABLE 1. In vitro stimulation of NK activity at different times of incubation

PBL treatment	% Specific ^{51}Cr release (E/T 10:1)			
	Expt 1		Expt 2	
	4 h[a]	18 h	4 h	18 h
None[b]	15 ± 1.3	36 ± 1.5	7 ± 1.6	44 ± 1.2
IFN[c]	24 ± 0.2	44 ± 1.5	12 ± 0.3	66 ± 0.5
Virion[d]	35 ± 1.5	64 ± 0.8	22 ± 0.3	81 ± 2.7
HA[e]	28 ± 0.5	47 ± 1.1	12 ± 0.1	61 ± 1.4
NA	24 ± 0.8	45 ± 3.5	13 ± 1.3	57 ± 2.3
HA-NA	24 ± 0.2	45 ± 1.4	12 ± 0.7	67 ± 1.8

[a] Incubation time of the assay system.
[b] Normal NK activity of PBL.
[c] IFN was used at 50 IU/ml.
[d] Purified influenza A/PC was used at 3×10^{-1} μg/ml.
[e] Virus glycoproteins HA and NA and the 1/1 mixture (combined concentration) were used at 11×10^{-1} μg/ml.

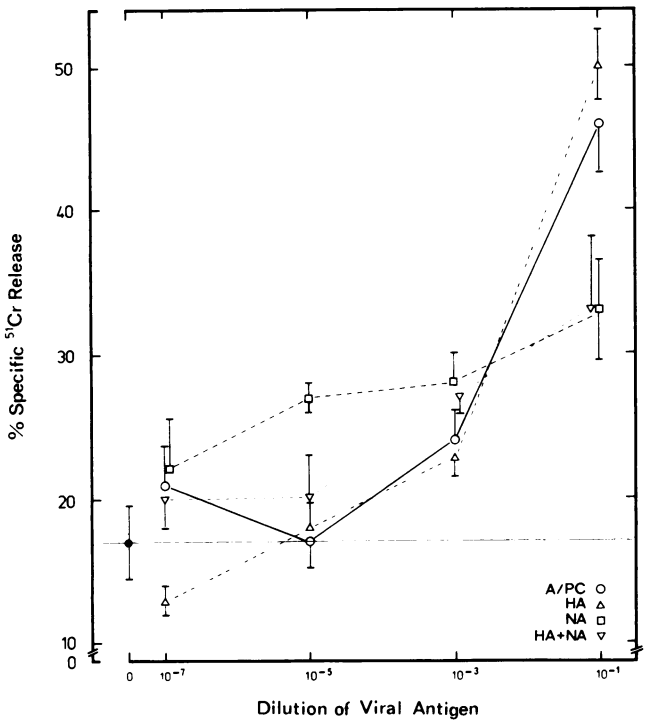

FIG. 1. Dose-response relationship. Ficoll-Hypaque-purified PBL and chromium-labeled K562 target cells (E/T 10:1) were exposed to four dilutions (10^{-1} to 10^{-7}) of intact whole virion (A/PC) or purified viral glycoproteins (HA, NA, and HA-NA), corresponding to concentrations 3×10^{-1} to 3×10^{-7} μg/ml and 11×10^{-1} to 11×10^{-7} μg/ml, respectively. The combined concentration of HA-NA represents half of each of HA and NA. The 0 value (18 ± 1.5%) represents normal NK activity when no virus is added to the test system. Vertical bars indicate standard deviations.

sion, virus, or virus glycoproteins for 30 min at 37°C before their addition to the incubation mixture of target and effector cells; or (ii) antiserum and antigen were added directly to the incubation mixture. Samples of 10 μl of a 10^{-1} dilution of antiserum were used.

The above experiments were repeated with the divalent antibody fragments $F(ab')_2$ of each antiserum. Samples of 10 μl (1.0 mg/ml) of $F(ab')_2$ were used.

RESULTS

Time course of virus-induced NK activity. Purified virion (A/PC, 3×10^{-1} μg/ml), HA or NA (11×10^{-1} μg/ml), HA plus NA (11×10^{-1} μg/ml, combined concentration), and IFN (50 IU/ml) were tested for in vitro stimulation of NK activity after 4 and 18 h of incubation of the assay system. The results of two independent experiments are presented in Table 1. In all experiments, a stimulation of NK activity was observed after 4 h. An increase of about twofold in the stimulation was apparent when the assay system was incubated for 18 h in experiment 1. A similar increase (about 4.5-fold) was observed between the two periods of incubation (4 and 18 h) in experiment 2. The stimulation induced due to the intact virion was highest (35 ± 1.5 and 22 ± 0.3 after 4 h; 64 ± 0.8 and 81 ± 2.7 after 18 h) followed by HA (28 ± 0.5 and 12 ± 0.1 after 4 h; 47 ± 1.1 and 61 ± 1.4 after 18 h). The performance of NA was similar to that observed for HA; the stimulation was 24 ± 0.8 and 13 ± 1.3 after 4 h and 45 ± 3.5 and 57 ± 2.3 after 18 h. The HA-NA mixture and IFN stimulated NK activity in a manner comparable to the individual performance of the glycoproteins. Since maximum NK activity was observed at 18 h, this period of incubation was used for experiments described next.

Dose-response curve. Different concentrations of purified influenza virus glycoproteins, 11×10^{-1} to 11×10^{-7} μg/ml (corresponding to dilutions 10^{-1} to 10^{-7} in Fig. 1), were used to determine the concentration needed to induce a maximum stimulation of NK activity. Purified intact virion (A/PC) preparations served as controls. A representative study is shown in Fig. 1. We repeatedly observed that only for the purified NA preparation, small doses, 11×10^{-5} to 11×10^{-7} μg/ml, were active. At these concentrations, an increase in NK activity from 22 to 28% was observed. Normal NK activity, at a ratio of E/T of 10:1, was 18 ± 2.0%. Increasing the concentration of NA to 11×10^{-1} μg/ml further increased NK activity to 32%. On the other hand, HA could stimulate NK activity to 24% only at 11×10^{-3} μg/ml; as the concentration of HA was increased, there was likewise an increase in NK activity. A maximum stimulation of NK activity (50%) was observed at 11×10^{-1} μg of HA per ml. Virus-induced NK stimulation was of the same order of magnitude as that observed for HA. It was interesting to note that the amount of purified influenza virus glycoproteins needed to stimulate NCMC to a level equivalent to that reached by untreated whole virions was almost four times higher, i.e., 11×10^{-1} μg versus 3×10^{-1} μg.

The above results demonstrate (i) that HA and NA are indeed capable of enhancing NK activity and (ii) that 11×10^{-1} μg of purified NA or HA per ml is the optimal concentration that induces maximal stimulation of NK activity. Detailed results obtained by in vitro exposure of PBL from eight different donors to virus preparations (complete or Formalin-inactivated virions) or with viral glycoproteins (HA, NA, or a mixture of both), are presented in Table 2. Both HA and NA stimulated NK activity in all donors to the same degree as that observed with whole virions (untreated or Formalin inactivated), confirming our previous observations (19a) that untreated influenza whole virions and inactivated vaccines, regardless of whether they are of type A or type B, could equally stimulate NK activity. It was of interest to note that the HA and NA mixture did not perform better than its components, either HA or NA. The results in Table 2 demonstrated that (i) 4 out of 10 experiments showed that the stimulation by HA was greater than that produced by the 1:1 mixture, as in Fig. 1, (ii) 4 out of 10 experiments indicated an equivalent level of stimulation of NK activity, and (iii) only 2 of 10 experiments showed the HA-NA mixture to have stimulated more than HA alone.

Treatment of virions with proteolytic enzymes. Since HA and NA might be isolated from influenza virions by digesting the virus particles with proteolytic enzymes (5, 12, 24, 28, 29, 34, 36, 38), this approach was used to remove the surface glycoproteins from purified virions. Results obtained with the pronase-, bromelain-, and chymotrypsin-treated virus in two independent experiments are presented in Table 3. Pronase treatment of purified virus particles removed 82% of the HA activity and 62% of the NA activity, and likewise the capacity to enhance NCMC (after appropriate correction for normal NK activity) was reduced by about 50% (Table 3). Treatment of virions with chymotrypsin and bromelain removed 99% of the HA activity and about 90% of the NA activity, and the capacity of these virus cores to stimulate the NK activity was also reduced by about 80%.

TABLE 2. In vitro stimulation of NK activity by influenza virus and its glycoproteins in individual donors

Donor	NK activity[a] with the following PBL treatment:					
	None[b]	Virions[c]		Glycoproteins[d]		
		Untreated	Formalin inactivated	HA	NA	HA-NA (1:1)
DM	28 ± 1.5	40 ± 1.3[e]		38 ± 0.4[e]	41 ± 1.1[e]	42 ± 0.5[e]
	17 ± 2.5	48 ± 0.6		36 ± 1.4	34 ± 2.7	32 ± 0.3
	12 ± 2.0	36 ± 2.4[f]	37 ± 1.1[f]			
	11 ± 1.1	29 ± 0.6[g]	30 ± 0.4[g]			
JB	23 ± 1.1	38 ± 2.6[e]		36 ± 1.0	33 ± 0.4	36 ± 3.3
	27 ± 1.9	46 ± 1.8		46 ± 0.4	49 ± 3.6	49 ± 0.7
	28 ± 1.3	41 ± 0.6[g]	41 ± 0.3[g]			
	21 ± 3.7	35 ± 0.5[h]	38 ± 1.6[h]			
	28 ± 3.5	57 ± 2.7[f]	52 ± 2.2[f]			
	23 ± 0.8	35 ± 0.5[g]	38 ± 1.6[g]			
RB	27 ± 2.6	40 ± 1.4[e]		39 ± 1.8	32 ± 2.5	40 ± 1.5
	26 ± 1.5	35 ± 3.5		35 ± 1.0	33 ± 1.6	36 ± 1.2
	28 ± 0.5	53 ± 3.1[h]	41 ± 1.2[h]			
AF	31 ± 1.4	49 ± 2.9[e]			49 ± 0.7	
	28 ± 0.3	40 ± 1.3[g]	41 ± 0.7[g]			
MS	28 ± 4.7	60 ± 0.5[e]	59 ± 0.7[g]	58 ± 0.2	54 ± 0.3	54 ± 0.1
	28 ± 2.1	59 ± 0.7	57 ± 3.2	59 ± 0.1	48 ± 0.2	49 ± 0.3
	21 ± 0.6	47 ± 0.7[h]	49 ± 0.9[h]			
MB	45 ± 0.1	58 ± 0.1[e]	59 ± 0.2[g]	60 ± 0.4	60 ± 0.2	58 ± 0.1
	48 ± 0.2	59 ± 1.2[h]	61 ± 1.3[h]			
LG	10 ± 3.0	25 ± 1.0[f]	26 ± 1.2[f]			
	14 ± 3.0	25 ± 3.9[g]	26 ± 1.3[g]			
PR	49 ± 1.2	63 ± 0.2[e]	68 ± 0.1[g]	64 ± 1.2	65 ± 0.1	63 ± 0.1
	43 ± 4.3	69 ± 1.2[f]	66 ± 2.2[f]			

[a] NK activity, (E/T 10:1).
[b] Normal NK activity of PBL.
[c] Intact, untreated virions or Formalin-inactivated virions were used at 3×10^{-1} μg/ml. The untreated virions were A/PC, and A/Brazil/11/78 (H1N1), A/Bangkok/1/79 (H3N2), and B/Singapore/222/79. The monovalent vaccines A/Brazil/, A/Bangkok, and B/Singapore were kindly supplied by Institut Armand-Frappier Production Inc.
[d] Virus glycoproteins HA and NA and the 1:1 mixture (combined concentration) were used at 11×10^{-1} μg/ml.
[e] NK activity stimulated by A/PC untreated virion or purified glycoproteins.
[f] NK activity stimulated by B/Singapore untreated virion or Formalin-inactivated virion.
[g] Same as in *f*, but with A/Bangkok.
[h] Same as in *f*, but with A/Brazil.

Inhibition of glycoprotein-induced CMC by monospecific antisera. To further demonstrate that the observed stimulation was indeed due to the glycoproteins, the following experiment was performed. Virus preparations were treated separately with each monospecific antiserum, anti-HA or anti-NA, before its effect on virus-induced NK activity was evaluated in the cytotoxicity assay. Complete inhibition of virus-induced CMC could be achieved in the presence of either anti-HA or anti-NA (Fig. 2A). The stimulation induced by purified HA preparations was, however, only suppressed in the presence of its homologous antiserum, i.e., anti-HA. The addition of anti-NA serum had no suppressive effect on HA-induced CMC (Fig. 2B). On the other hand, NA-stimulated NCMC could be suppressed in the presence of its homologous anti-NA serum as well as in the presence of anti-HA antiserum (Fig. 2C). These results were obtained either by the preincubation of antisera and antigen or by the direct addition of these preparations simultaneously to the test system.

Similar results were obtained when $F(ab')_2$ fragments of each antiserum were used, thus confirming the results obtained with the monospecific antisera.

None of the monospecific antisera or $F(ab')_2$ fragments used had any effect on either normal or IFN-activated NK activity (data not shown).

DISCUSSION

This report identifies the viral components responsible for influenza virus-induced CMC. Three different approaches were used. The combined results of these experiments conclusively demonstrate that influenza virus surface glycoproteins HA and NA can strongly stimulate NK activity of human PBL against K562 target cells.

First, results from short-term experiments (Table 1), wherein HA and NA were tested individually for their potential to stimulate early virus-induced CMC, demonstrated significant boosted NK activity as soon as 4 h after incubation. The dose-response curves for viral glycoproteins either in purified form or in association with the virion were equivalent in terms of NCMC induction. At low concentrations, however, only NA was a significant stimulator of NCMC; HA, like whole virions, was an effective stimulator at higher concentrations. The results (Fig. 1) obtained with an equivalent mixture of HA-NA (1:1)-purified glycoproteins

TABLE 3. Effect of treatment of influenza virions with proteolytic enzymes

Expt[a]	Treatment of virion[b] (μg/ml)	HA activity (%)	NA activity (%)	% Specific ^{51}Cr release (E/T 10:1)
1	Untreated	100.0	100.0	34 ± 0.1[c]
	Pronase (1.0)	20.0	36.1	28 ± 1.5
	Bromelain (15)	0	6.6	25 ± 0.2
	Chymotrypsin (0.024)	0	11.8	21 ± 2.6
2	Untreated	100.0	100.0	60 ± 2.9
	Pronase (1.0)	16.0	39.8	49 ± 1.1
	Bromelain (15)	2.0	4.2	41 ± 0.1
	Chymotrypsin (0.024)	2.0	6.2	38 ± 2.4

[a] Treatment of purified influenza A/PC virion (10 μg/ml) with proteolytic enzymes was at 37°C for 60 min.

[b] Normal NK activity (E/T 10:1) was 17 ± 3.5% in experiment 1 and 37 ± 0.2% in experiment 2. In these experiments PBL were obtained from two different donors.

[c] Mean value of triplicate samples ± standard deviation.

showed that the NK activity due to each glycoprotein was independent of the presence of the other, as no additive effect was observed. The results reported in Table 2, however, showed that in 2 out of 10 experiments the NK activity stimulated by the HA-NA mixture was superior to that observed for HA alone. The reasons for the discrepancy remain unanswered. However, should the NK stimulation by HA and NA alone be independent processes, then it is possible that HA and NA enhance CMC via the same cellular site. This argument is appealing because the virus-induced activity can be completely inhibited with either anti-HA or anti-NA antibody. Alternatively, the apparent lack of additive enhancement may be due to differences in the boosting activity of the two glycoproteins used, as they probably differ in the concentrations needed for optimal enhancement. The concentration of purified glycoproteins required to achieve a stimulation equivalent to that observed for whole virion preparations was almost fourfold greater. This observation was somewhat different from that reported by Casali et al. (7) who reported that only 20% of the purified glycoproteins of measles virus (HA and fusion) were required to match the NK activity observed for measles whole virion preparations.

Second, when virions were treated with proteases such as pronase, bromelain, and chymotrypsin, viral cores lacking either HA activity or both HA and NA activity (although the latter could not be removed completely) showed that NK activity was related to the presence of HA and NA proteins. The removal of both glycoproteins by bromelain and chymotrypsin led to 90% inhibition of virus-induced CMC. However, pronase-treated virions retained 40% of their NA activity, and the NK activity induced was approximately of the same order. This again suggests that the stimulation of NK activity by the two glycoproteins is not additive.

Finally, treatment of HA and NA antigens with their homologous monospecific antisera markedly reduced NK activity, suggesting again the active role played by these two proteins in enhancing NK activity. That is, treatment of HA

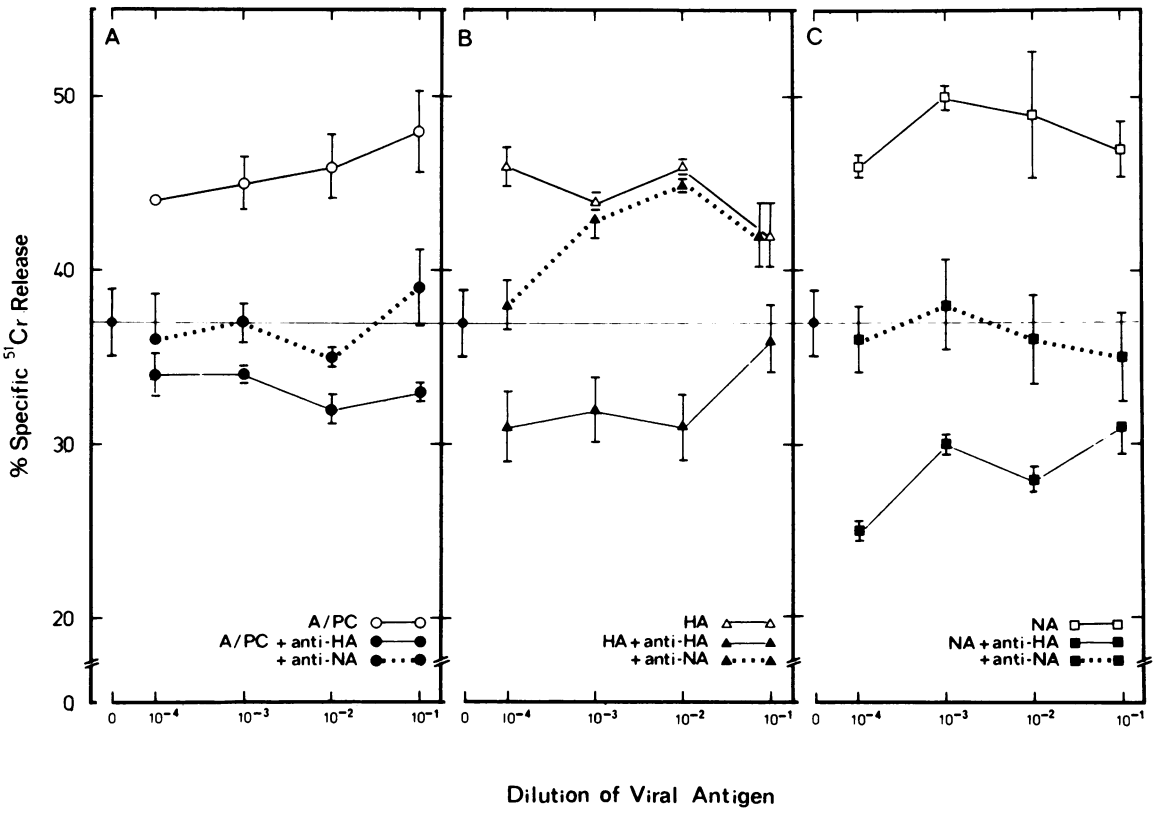

FIG. 2. Effect of monospecific antisera on virus-induced NCMC. A 10^{-1} dilution of each monospecific antiserum was added directly to the test system to demonstrate its effect on (a) virus-induced CMC, (b) HA-induced CMC, and (c) NA-induced CMC. Tenfold dilutions of the virus antigens, whole virion (A/PC), and each of the viral antigens HA and NA were used. A 10^{-1} dilution of viral antigen represents a protein concentration of 3×10^{-1} μg/ml (virus) or 11×10^{-1} μg/ml (HA and NA). The 0 value (38 ± 2.5%) represents normal NK activity.

with homologous monospecific antiserum (anti-HA) demonstrates the specificity of influenza virus-induced CMC. However, it was interesting to note that the stimulated NK activity due to NA antigen could be suppressed by the addition of anti-HA in the test assay. It appeared that, although the anti-HA was a monospecific antiserum when verified by immunodiffusion and HA and NA inhibition tests, this serum contained a component(s) that reacted with the active site of the NA protein responsible for the induction of NK activity. Cross-reactivity of antibodies specific for NA with the HA glycoprotein has been previously reported (20, 35). The stimulatory potential of each glycoprotein may be reduced by steric hindrance (7). The complete inhibition of virus-induced NK activity by each of the two monospecific antisera (anti-HA and anti-NA) suggests that HA and NA probably share the same cellular site in stimulating CMC. This and other possibilities remain to be tested.

Since the presence of the Fc portion of the immunoglobulin molecule might interfere with the stimulation of NK activity under the experimental conditions used, the validity of the results reported with monospecific antisera was verified by using the $F(ab')_2$ fragments isolated from each antiserum. The results confirmed the contention that the observed stimulation was, indeed, glycoprotein-induced CMC. These results are in agreement with our previous observations with influenza virus (19a) and with those reported in other virus systems, such as with mumps (19) and Sendai (1) viruses, that identified the HA-NA as essential in the induction of CMC.

With regard to the mechanism of virus-induced CMC, several studies have demonstrated that human NK activity directed against virus-infected or virus-exposed targets can follow an IFN-independent route. These results include the following. (i) Peak NK cytotoxicity, at 4 to 6 h, precedes the peak of IFN production, and NK cytotoxicity and IFN production are thus not temporally correlated (4, 7, 9, 17). (ii) Casali et al. (7) observed a stimulation of NK activity against both fibroblast and K562 targets with purified measles virus glycoproteins without the concomitant release of IFN, yet whole virus and UV-inactivated virus did activate CMC with IFN production. (iii) NK activity was stimulated by one of the two glycoproteins of the mumps (19, 37) and Sendai (1) viruses, with no detectable IFN production; furthermore, virions devoid of HA did not enhance CMC. (iv) Pretreatment of NK cells with exogenous IFN increases NK activity against both uninfected and virus-infected or virus-exposed target cells (4, 16). These studies support the concept that NK activity and IFN production are independent processes and probably coexist and operate independently.

It is accepted that both IFN-independent and IFN-dependent mechanisms can operate within a single virus system. Early CMC (less than 4 h) is IFN independent with respect to stimulation by virus glycoproteins, whereas later CMC (after 8 h) is IFN dependent (1, 4, 7, 37). Since an 18-h incubation of the assay system was used in the present study, one can argue for the production of IFN and thus conclude that the observed induced CMC is IFN dependent. Since no IFN was detected in the assay system used, the results suggest that the influenza glycoprotein-induced CMC is an IFN-independent process, but further studies are needed to unveil the exact mechanisms involved.

Results further suggest that viral replication is not necessary for the mediation of virus-induced CMC, as reported earlier with measles virus (7), because the NK stimulation pattern achieved with inactivated virions was identical to that observed with its untreated counterparts or to those elucidated with purified HA and NA glucoproteins.

ACKNOWLEDGMENTS

This investigation was supported under National Health Research and Development Project no. 6605-1718-S4 of the Department of National Health and Welfare, Canada, and Natural Sciences and Engineering Research Council of Canada, grant no. A8371.

LITERATURE CITED

1. **Alsheikhly, A., C. Orvell, B. Harfast, T. Andersson, P. Perlmann, and E. Norrby.** 1983. Sendai-virus-induced cell-mediated cytotoxicity in vitro. The role of viral glycoproteins in cell-mediated cytotoxicity. Scand. J. Immunol. **17**:129–138.
2. **Arora, D. J. S., J. Hill-Schubert, and L. Vincent.** 1980. The presence of two neuraminidases in an influenza virus. Can. J. Microbiol. **26**:243–249.
3. **Arora, D. J. S., V. Pavilanis, and P. Robert.** 1973. Two-step centrifugation method. A simplification of the density-gradient procedure for the purification of influenza virus. Can. J. Microbiol. **19**:633–638.
4. **Bishop, G. A., J. C. Glorioso, and S. A. Schwartz.** 1983. Role of interferon in human natural killer activity against target cells infected with HSV-1. J. Immunol. **131**:1849–1853.
5. **Brand, C. M., and J. J. Skehel.** 1972. Crystalline antigen from the influenza virus envelope. Nature (London) New Biol. **238**:145–147.
6. **Brunda, M. J., R. B. Herberman, and H. T. Holden.** 1980. Interferon-independent activation of murine natural killer cell activity, p. 525–532. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
7. **Casali, P., J. G. P. Sissons, M. J. Buchmeir, and M. B. A. Oldstone.** 1981. In vitro generation of human cytotoxic lymphocytes by virus. Viral glycoproteins induce non-specific cell-mediated cytotoxicity without release of interferon. J. Exp. Med. **154**:840–855.
8. **Ching, C., and C. Lopez.** 1979. Natural killing of herpes simplex virus type 1-infected cells: normal human responses and influence of antiviral antibody. Infec. Immun. **26**:48–56.
9. **Copeland, C. S., H. S. Koren, and P. J. Jensen.** 1981. Natural killing can be independent of interferon generated in vitro. Cell. Immunol. **62**:220–225.
10. **Couch, R. B., and J. A. Kasel.** 1983. Immunity to influenza in man. Annu. Rev. Microbiol. **37**:529–549.
11. **Djeu, J. Y., N. Stocks, K. Zoon, G. J. Stanton, T. Timonen, and R. B. Herberman.** 1982. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes infection. J. Exp. Med. **156**:1222–1234.
12. **Drzeniek, R., J. T. Seto, and R. Rott.** 1966. Characterization of neuraminidases from myxoviruses. Biochim. Biophys. Acta **128**:547–558.
13. **Ennis, A.** 1982. Some newly recognized aspects of resistance against and recovery from influenza. Arch. Virol. **73**:207–217.
14. **Ennis, F. A., and A. Meager.** 1981. Immune interferon produced to high levels by antigenic stimulation of human lymphocytes with influenza virus. J. Exp. Med. **154**:1279–1289.
15. **Ennis, F. A., A. Meager, A. S. Beane, Q. Yi-Hua, D. Riley, G. Schwartz, G. C. Schild, and A. H. Rock.** 1981. Interferon induction and increased natural-killer cell activity in influenza infections in man. Lancet **ii**:891–893.
16. **Fitzgerald, P. A., R. Evans, D. Kirkpatrick, and C. Lopez.** 1983. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1-infected fibroblasts and K562 erythroleukemia targets. J. Immunol. **130**:1663–1667.
17. **Fitzgerald, P. A., P. von Wussow, and C. Lopez.** 1982. Role of endogenous and exogenous interferon in natural kill of HSV-1 infected fibroblasts. J. Immunol. **129**:819–823.
18. **Haller, O.** 1981. Inborn resistance of mice to orthomyxoviruses.

Curr. Top. Microbiol. Immunol. **92**:25–52.
19. **Harfast, B., C. Orvell, A. Alsheikhly, T. Andersson, P. Perlmann, and E. Norrby.** 1980. The role of viral glycoproteins in mumps virus-dependent lymphocyte-mediated cytotoxicity in vitro. Scand. J. Immunol. **11**:391–397.
19a. **Justewicz, D. M., J. Lecomte, and R. Mandeville.** 1984. Blocking of influenza virus-induced cell-mediated cytotoxicity by hemagglutinin-specific monoclonal antibody. J. Infect. Dis. **150**:348–357.
20. **Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster.** 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J. Virol. **2**:281–288.
21. **Laver, W. G.** 1969. Purification of influenza virus, p. 82–86. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press, Inc., New York.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Mandeville, R., M. F. Sombo, and N. Rocheleau.** 1983. Natural cell mediated cytotoxicity in normal human peripheral blood lymphocytes and its in vivo boosting with BCG. Cancer Immunol. Immunother. **15**:17–22.
24. **Nermut, M. V.** 1972. Further investigation on the fine structure of influenza virus. J. Gen. Virol. **17**:317–331.
25. **Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. L. Woernley.** 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulphide bonds. Arch. Biochem. Biophys. **89**:230–244.
26. **Potter, C. W., and J. S. Oxford.** 1979. Determinants of immunity to influenza in man. Br. Med. Bull. **35**:69–75.
27. **Santoli, D., and H. Koprowski.** 1979. Mechanisms of activation of human natural killer cells against tumor and virus infected cells. Immunol. Rev. **44**:125–163.
28. **Schulze, I. T.** 1970. The structure of influenza virus. I. The polypeptides of the virion. Virology **42**:890–904.
29. **Seto, J. T., R. Drzeniek, and R. Rott.** 1966. Isolation of a low molecular weight sialidase (neuraminidase) from influenza virus. Biochim. Biophys. Acta **113**:402–404.
30. **Stein-Streilein, J., M. Bennett, D. Mann, and V. Kumar.** 1983. Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection. J. Immunol. **131**:2699–2704.
31. **Timonen, T., J. R. Ortaldo, and R. B. Herberman.** 1981. Characterization of human large granular lymphocytes and relationship to natural killer and K cell. J. Exp. Med. **153**:569–582.
32. **Timonen, T., E. Saksela, I. Virtanen, and K. Centell.** 1980. Natural killer cells are responsible for the interferon production induced in human lymphocytes by tumor cell contact. Eur. J. Immunol. **10**:422–427.
33. **Trinchieri, G., D. Santoli, R. R. Dee, and B. B. Knowles.** 1978. Antiviral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the antiviral activity as interferon and characterization of the human effector lymphocyte subpopulation. J. Exp. Med. **147**:1299–1313.
34. **Varghese, J. N., W. G. Laver, and P. M. Colman.** 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature (London) **303**:35–40.
35. **Webster, R. G., V. S. Hinshaw, and W. G. Laver.** 1982. Selection and analysis of antigenic variants of the neuraminidase of N2 influenza viruses with monoclonal antibodies. Virology **117**:93–104.
36. **Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild.** 1982. Molecular mechanisms of variation in influenza viruses. Nature (London) **296**:115–121.
37. **Welsh, R. M.** 1981. Natural killer cells in virus infection. Curr. Top. Microbiol. Immunol. **92**:83–106.
38. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane and glycoproteins of influenza virus at 3A resolution. Nature (London) **289**:366–373.
39. **Yasukawa, M., and M. J. Zarling.** 1983. Autologous herpes simplex virus-infected cells are lysed by human natural killer cells. J. Immunol. **131**:2011–2016.