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Antineuraminidase Antibody Response to Vaccination of Chickens with Intact Virus and Different Subunit Preparations of the Influenza Virus Strains A/Sing/1/57 (H2N2), A/Hong Kong/1/68 (H3N2) and A/Port Chalmers/1/73 (H3N2)*

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Summary

The antineuraminidase (AN) antibody response to vaccination of chickens with intact virus and different subunit preparations of the influenza virus strains A/Sing/1/57 (H2N2), A/Hong Kong/1/68 (H3N2) and A/Pt. Chalmers/1/73 (H3N2) was tested comparatively.

Using a photometric method capable of analysing mixtures of AN antibodies against antigenically different N2 neuraminidases, it was concluded that vaccination with subunits produced by treatment with bromelain and Sarkosyl can yield AN antibody response against heterologous neuraminidase. By contrast, vaccination with intact and ether-treated virus gave AN antibody response against homologous neuraminidase, only.

These findings were confirmed when testing sera by means of enzyme inhibition test and by adsorption experiments with homologous and heterologous neuraminidases.

The conclusion was reached that the NA's of the strains A/Sing/1/57 and A/Pt. Chalmers/1/73 share antigenic determinants and that the NA of the strain A/Hong Kong/1/68 shares antigenic determinants with that of the strains A/Sing/1/57 and A/Pt. Chalmers/1/73.

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Abbreviations used: ACU = antibody concentration unit; AN = antineuraminidase; HA = hemagglutinin; HCU = hemagglutinin concentration unit; mU = micro units; NA = neuraminidase; NIT = neuraminidase inhibition test; PBS = 0.15 M NaCl solution buffered at pH 7.0 with 0.01 M phosphate.

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Introduction

The hemagglutinins (= HA's) and neuraminidases (= NA's) of influenza viruses show independent antigenic variation (20, 23) which has been assumed to result from recombination between strains of human and animal origin (15, 23).

Another possible explanation is to assume that antigenic variation occurs by rearrangement of antigenic components common to a family of strains and that some of these antigens occupy an inaccessible subsurface position (4).

If this were so, disruption of virus could unmask such antigens.

This has been repeatedly described for the HA's of different influenza virus A strains produced by ether-treatment (4, 10).

Furthermore, it has been previously reported (5) that influenza virus N2 NA's isolated by use of bromelain-treatment can differ antigenically from the NA present on intact virus.

Therefore, the experiments described in this paper were designed in order to examine whether or not vaccination of chickens with viral subunits produced by different techniques can yield AN antibody response against heterologous NA.

The NA's of influenza virus H2N2 and H3N2 strains have been reported to be antigenically inhomogeneous (6, 12, 17).

The antigenic relationship between N2 NA's has been examined by means of a photometric hemagglutination inhibition test (ACU-test) (8), using recombinants possessing a relevant NA and an irrelevant HA (12).

It was found that three classes of N2 NA, designated NA_a, NA_b and NA_c can be defined: NA_a is represented by the NA of the strain A/Sing/1/57 and other H2N2 strains, NA_b by the NA of A/Hong Kong/1/68 virus and NA_c by the NA of A/Pt. Chalmers/1/73 virus (12). This classification agrees with previous reports (17).

Each class of AN antibodies gave different titer ratios when allowed to react with the recombinants A/Bel/34 (H0) — A/Sing/57 (N2) (= Bel-Sing), A/equine—Prague (Heq1) — A/Hong Kong/1/68 (N2) (= X15—HK) and mixtures of both recombinants (12).

It has been found that recording these titer ratios for reference sera and test sera can be utilized to determine the concentration of the different classes of AN antibodies in the sera to be tested (12).

This technique has been found to give reliable results when used for analysing artificially prepared mixtures of AN antibodies (12).

This method was used for recording comparatively the AN antibody response to vaccination with intact virus and different subunit preparations of the influenza virus strains A/Sing/1/57 (H2N2), A/Hong Kong/1/68 (H3N2) and A/Port Chalmers/1/73 (H3N2). In addition, sera were tested by means of NIT (1) and by examining the influence of adsorption with homologous and heterologous NA on their AN titers.

Materials and Methods

Virus

The strains of egg-adapted non-inactivated influenza virus employed were:

A/Sing/1/57 (H2N2) (= A/Sing), A/Hong Kong/1/68 (H3N2) (= A/HK), A/Pt. Chalmers/1/73 (H3N2) (= A/PC) and the recombinants A/Bel/34 (H0)—A/Sing/1/57

(N 2) (= Bel-Sing) A/equine—1/Prague/56 (Heq 1)—A/Hong Kong/1/68 (N 2) (= ×15-HK), and A/equine—1/Prague/56 (Heq 1)—A/Port Chalmers/1/73 (N 2) (= eq.-PC).

Virus was purified by adsorption onto and elution from $BaSO_4$ (7) and subsequently by several steps of differential centrifugation and by sucrose-gradient ultracentrifugation (14). Virus was suspended in 0.15 M NaCl solution, buffered at pH 7.0 with 0.01 M phosphate (= PBS). The HA titers were determined by use of the photometric HCU method and were expressed in terms of HCU units (7).

Nitrogen Assay

The nitrogen content of the samples was assayed by means of a Kjeldahl microtechnique as described previously (5).

For the virus strains used, the ratios of HCU's per γ_N ranged from 173 to 477 and the ratios of NA to HCU from 0.0121 to 0.0286 mU/HCU.

Ether-Treatment of Virus Suspensions

The technique employed was that previously described (7, 9). In brief, mixtures of one volume of virus suspension and of three volumes of peroxide-free ether were incubated for 8 hours at 4° C. After overnight separation by holding the mixture at 4° C in a funnel, the aqueous phase was reextracted and excess ether was removed with N_2.

Disruption of Virus with Bromelain

Virus was disrupted with bromelain under reducing conditions as described by BACHMAYER *et al.* (2). In brief, purified virus was suspended in Tris-HCl buffer (0.1 M, pH 7.2) containing 3 mg/ml of bromelain (Schuchardt GmbH and Co., München, Federal Republic of Germany), 0.05 M 2-mercaptoethanol (Fluka A.G., Buchs, Switzerland) and 0.001 M EDTA (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany). After 16 hours of incubation at 37° C, suspensions were fractionated by ultracentrifugation at $150{,}000 \times g$ for 60 minutes. After removal of the supernatants, the pellets were resuspended in Tris-HCl buffer. The supernatants and the resuspended sediments were dialysed for 48 hours at 4° C against PBS.

Sarkosyl Treatment of Virus

Purified virus was disrupted by means of Sarkosyl as described by STANLEY *et al.* (21). In brief, to 40,000 to 100,000 HCU's of purified virus 5 per cent (w/v) of Sarkosyl NL 97 (kindly supplied by the Ciba/Geigy A.G., Wehr/Baden, Germany) were added and the mixture was incubated at 37° C for 30 minutes.

Subsequently, undisrupted virus and core material was pelleted by centrifugation at $90{,}000 \times g$ for 60 minutes. The supernatant was carefully withdrawn and the sediment resuspended in PBS. The supernatant was dialysed against 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.1 per cent Sarkosyl.

Neuraminidase Determination

The activity of NA was assayed by use of the method recommended by the WHO (1), using fetuin purchased from K and K Labs., Plainview, New York, as substrate. The NA activity was expressed in terms of mU/ml (1).

Designation of Subunit Preparations

The symbol "E" was used for ether-treated virus and the symbols "B" and "S" to indicate that subunits were obtained by treatment with bromelain or Sarkosyl. The supernatant fractions were "Sup" and the sediment fractions "Sed".

Vaccination Experiments

Three series of experiments with subunit vaccines and the corresponding intact virus vaccines (one series with A/Sing, the second with A/HK and the third with A/PC virus vaccines) were carried out. In a fourth series of experiments, chickens received monovalent vaccines containing graded amounts of intact virus of the three strains employed. Three 8 to 9-week-old chickens were used in each experiment.

Primary vaccination consisted of a 2 ml—dose of water-in-oil emulsified vaccine (incomplete Freund's adjuvant purchased from Difco Labs., Detroit) given intramuscularly. Subsequent vaccinations were carried out by inoculating animals intraperitoneally with the same antigen doses as used for primary vaccination, suspended in 20 ml of PBS without addition of an adjuvant. The doses of NA given per animal were expressed in terms of mU of NA.

Unless otherwise stated, animals were vaccinated on day 1 and 28. The vaccines were stored at —20° C before use. Blood samples were drawn by heart puncture. Aliquots of the sera of each group were pooled and treated with M/90 KJO_4. In each series of experiments, primary vaccination was carried out on the same day and all sera were tested in parallel for AN antibodies.

Titration of AN Antibodies by Means of the Photometric ACU Method

The AN antibody titers against the NA of the recombinant Bel-Sing ($= NA_a$), X15-HK ($= NA_b$) and eq.-PC ($= NA_c$) were determined as follows:

As first step, the reciprocal highest serum dilution (d_{50}) yielding binding of 50 per cent of 90 to 110 HCU's of each recombinant were determined as described previously (11) and the d_{50} values found were designated D (reaction with NA_a), D′ (reaction with NA_b) and D″ (reaction with NA_c).

As second step, the percentages (a, b, and c) of AN antibodies against NA_a (= a), NA_b (= b) and NA_c (= c) were determined by means of the method for analysis of AN antibody mixtures (12). Values not differing significantly ($P < 0.01$) from 0.01 were scored as 0.

As third step, the titers of AN antibodies against NA_a ($= ACU_{AN,a}$), NA_b ($= ACU_{AN,b}$) and NA_c ($= ACU_{AN,c}$) were calculated as follows:

1. $ACU_{AN,a} = a \cdot D$
2. $ACU_{AN,b} = b \cdot D \cdot n$
3. $ACU_{AN,c} = c \cdot D \cdot o$

n equals the geometric means of d_{50} values against X15-HK of sera produced by vaccination with intact A/HK virus divided by d_{50} values against Bel-Sing ($n = 17.26$) and

o equals the geometric means of d_{50} values against eq.-PC of sera produced by vaccination with intact A/PC virus divided by d_{50} values against Bel-Sing ($o = 3.57$).

Multiplication with n and o is necessary in order to convert titers (e.g. $b \cdot D$ and $c \cdot D$) pertaining to the reactivity of antibodies with Bel-Sing (12) into titers pertaining to the use of homologous recombinants.

The d_{50} values and ACU_{AN} titers presented in the subsequent sections are geometric means of 8 to 12 determinations.

Titration of AN Antibodies by Means of NIT-Tests

Titrations were carried out as recommended by the WHO (1). Titers were expressed in terms of the reciprocal highest serum dilution inhibiting 50 per cent of the starting NA activity.

Adsorption Experiments

Supernatants of sera adsorbed with the recombinants Bel-Sing and eq.-PC were prepared as described by LAVER *et al.* (16). In brief, sera were mixed with an excess of the recombinants, and unbound virus and the resulting virus—antibody complexes were separated from the unbound antibody by means of ultracentrifugation. The supernatants were removed and tested against the recombinants Bel-Sing and eq.-PC by means of the photometric ACU method (12).

Statistical Evaluation

The logarithms of the values of the fractions of AN antibodies (a, b and c) were found to be normally distributed and their standard deviations did not exceed 0.06 log units.

Using a t-test (22), the logarithms of fractions representing homologous antibody (e.g. values of a of sera produced by vaccination with A/Sing virus) were tested for significant differences from log 1 (= 0) and the logarithms of fractions representing heterologous antibody (e.g. values of b and c of sera prepared by vaccination with A/Sing virus) for significant differences from log 0.01 (= −2). Significantly ($P < 0.01$) differing values were underlined. The calculation of regression and correlation coefficients was carried out as described by WEBER (22).

Results

Yield of Detectable NA Activity Found in Subunit Preparations

Representative examples of the yields of detectable NA activity found when preparing viral subunits are listed in Table 1.

In comparison to the other strains, bromelain-treatment of the strain A/PC resulted in drastic reduction of the NA yield (B, Sup.). This has been described previously (3) for the NA of another recent H3N2 strain. In many cases, less than 100 per cent of the NA activity of the starting materials were found after disruption of virus.

It is very likely that subunit preparations contained variable amounts of NA which was no longer biologically active but was still antigenic.

Table 1. *Representative examples of the yields of NA activity found in subunit preparations*

Subunit preparation	Yield of NA activity[a] for strain		
	A/Sing	A/HK	A/PC
Ether-treated	77.1	42.7	56.1
Bromelain, supernate	99.7	100.0	5.9
Bromelain, sediment	4.2	3.0	0.4
Sarkosyl, supernate	59.0	108.1	62.4
Sarkosyl, sediment	1.9	3.1	13.7

[a] In per cent of the activity present in the starting material

Testing of the AN Antibody Response by Use of the ACU Test

Table 2 presents examples of the AN antibody response to vaccination with intact virus and subunit preparations as measured by means of ACU test.

The d_{50} values obtained when testing sera with the recombinants Bel-Sing, X15-HK and eq.-PC are listed in columns 6 through 8. Columns 9 through 11 give the fractions of AN antibodies (a, b and c) and columns 12 through 14 the ACU_{AN} titers against the NA's of the recombinants identified at the head of these columns.

Intact and ether-treated virus had the same and the other subunit preparations a lower antigenicity.

The conclusion was reached that vaccination with intact and ether-treated virus (Exper. 1, 2, 7, 8, 13 and 14) yielded ACU_{AN} titers against homologous NA, only.

By contrast, vaccination with subunits gave ACU_{AN} titers against heterologous NA:

Table 2. *Neuraminidase antibody response in chickens to vaccination with intact virus and subunit preparations. Results of ACU-tests*

1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Animals vaccinated with		Sera drawn days after 1st vaccin.[a]	Total amount of NA given per animal	d_{50} values measured with recombinant			Fractions of AN antibodies against Bel-Sing (= a), X 15-HK (= b) and eq.-PC (= c)			ACU_{AN} titers against recombinant		
Exp. No.	Strain	Preparation			Bel-Sing	X 15-HK	eq.-PC	a	b	c	Bel-Sing	X 15-HK	eq.-PC
1	A/Sing	Intact virus	35	24.2	639	937	124	1.00	0.00	0.00	639	0	0
2		Ether-tr. virus	35	25.8	521	1403	111	0.93	0.07	0.00	484	0	0
3		B, Sup.	119[b]	132.4	307	579	150	0.91	0.00	0.09	279	0	0
4		B, Sed.	100[c]	5.8	220	626	278	*0.68*[e]	0.00	*0.32*	150	0	251
5		S, Sup.	35	27.6	79	142	39	0.91	0.00	0.09	72	0	0
6		S, Sed.	35	2.2	215	332	145	0.88	0.00	*0.12*	189	0	92
7	A/HK	Intact virus	35	58.8	472	8145	358	0.00	1.00	0.00	0	8145	0
8		Ether-tr. virus	35	25.8	132	2262	96	0.00	1.00	0.00	0	2262	0
9		B, Sup.	35	59.0	235	1224	94	*0.75*	*0.24*	0.01	176	973	0
10		B, Sed.	47[d]	0.6	81	99	25	*0.98*	*0.00*	0.02	79	0	0
11		S, Sup.	35	27.6	124	1196	111	*0.39*	*0.49*	*0.12*	48	1048	53
12		S, Sed.	31	19.0	115	1546	114	*0.16*	*0.73*	*0.11*	18	1448	45
13	A/PC	Intact virus	35	5.2	159	866	567	0.00	0.00	1.00	0	0	567
14		Ether-tr. virus	35	2.8	148	769	518	0.03	0.00	0.97	0	0	512
15		B, Sup.	35	0.32	92	52	52	*0.96*	0.00	*0.04*	88	0	0
16		B, Sed.	47[d]	0.06	54	56	25	*0.96*	0.00	*0.04*	52	0	0
17		S, Sup.	38	17.8	73	186	212	*0.31*	0.00	*0.69*	23	0	180
18		S, Sed.	38	9.8	164	842	396	*0.28*	0.07	*0.65*	46	0	380

[a] Unless otherwise stated, animals were vaccinated on day 1 and 28
[b] Additional vaccinations on days 41, 45, 48, 51 and 66
[c] Additional vaccinations on days 53, 64, 68, 71, 74 and 89
[d] Additional vaccination on day 41
[e] Italic values differed significantly ($P < 0.05$) from 1.0 (tests with homologous NA) and 0.01 (tests with heterologous NA)

Vaccination with the sediment fractions of bromelain and Sarkosyl-treated A/Sing virus (Exper. 4 and 6) and with the supernatant and sediment fraction of Sarkosyl-treated A/HK virus (Exper. 11 and 12) resulted in ACU_{AN} titers against the NA of the recombinant eq.-PC.

The sera obtained by vaccination with the subunits produced by bromelain and Sarkosyl-treatment of strains A/HK and A/PC were found to contain ACU_{AN} titers against the NA of Bel-Sing (Exper. 9 through 12 and 15 through 18).

These findings were confirmed in at least two further analogous experiments with the following exceptions: In one experiment, vaccination with the sediment fraction of bromelain treated A/Sing virus yielded ACU_{AN} titers against homologous NA, only. However, the dose of NA injected per animal was much lower (1.13 mU) than in other experiments (5.8 mU).

In 4 of 9 experiments carried out with the supernatants of Sarkosyl-treated A/HK virus, ACU_{AN} titers against both Bel-Sing and eq.-PC were observed. In two experiments, ACU_{AN} titers against Bel-Sing but not against eq.-PC and in two further experiments ACU_{AN} titers against eq.-PC but not against Bel-Sing were found. In one experiment, ACU_{AN} titers against homologous NA, only, were recorded.

The doses of NA injected per animal were different in experiments vaccinated with intact virus and with subunits. Therefore, it was examined whether or not the finding of heterologous ACU_{AN} titers in experimental groups vaccinated with subunits could be due to the use of different amounts of antigen in comparison to the groups given intact virus.

Therefore, additional groups of animals were vaccinated with monovalent vaccines containing graded doses of intact viruses of the strains A/Sing, A/HK and A/PC. The NA doses injected per animal ranged from 0.2 to 150 mU. Blood samples were drawn 35, 45, 60 and 90 days after primary vaccination.

In no experiment, heterologous ACU_{AN} titers were found. This rules out that the finding of such titers in sera of groups vaccinated with subunits was caused by differences in the amount of antigen employed when vaccinating animals with intact virus and with subunits.

Testing of the AN Antibody Response by Use of NIT

The NIT-titers are given in columns 4 through 6 of Table 3. When comparing the ACU_{AN} titers and the NIT titers of sera, the following conclusions were reached:

i. Sera without ACU_{AN} titers against heterologous NA were found to have higher NIT titers with homologous NA than with heterologous NA. By contrast, many sera with ACU_{AN} titers against heterologous NA showed higher NIT titers with the heterologous NA than with the homologous NA (Exper. 4 and 12, reaction with eq.-PC, Exper. 9 through 12, 15 and 16, reaction with Bel-Sing.)

ii. For each serum, the NIT titer measured with heterologous NA was divided by the NIT titer obtained with homologous NA. All sera with ACU_{AN} titers against heterologous NA were found to give higher values of this ratio for the use of the heterologous NA than did the control sera obtained by vaccination with intact virus.

Table 3. *Neuraminidase antibody response in chickens to vaccination with intact virus and subunit preparations. Results of NIT*

1	2	3	4	5	6	7	8	9
	Animals vaccinated with		NIT-titers against recombinant			NIT-titer ratios		
Exp. No.	Strain	Preparation	Bel-Sing	X 15-HK	eq.-PC	Bel-Sing homol. NA	X 15-HK homol. NA	eq.-PC homol. NA
1	A/Sing	Intact virus	360	43	<40	*1.00*[a]	0.12	<0.11
2		Ether-tr. virus	320	40	<40	*1.00*	0.12	<0.12
3		B, Sup.	200	40	80	*1.00*	0.20	0.40
4		B, Sed.	80	40	120	*1.00*	0.50	*1.50*
5		S, Sup.	50	<40	<40	*1.00*	<0.80	<0.80
6		S, Sed.	150	<40	80	*1.00*	<0.27	*0.53*
7	A/HK	Intact virus	220	340	180	0.65	*1.00*	0.53
8		Ether-tr. virus	60	110	50	0.54	*1.00*	0.45
9		B, Sup.	110	65	<40	*1.69*	*1.00*	<0.61
10		B, Sed.	50	<40	<40	>*1.25*	—[b]	—
11		S, Sup.	90	70	48	*1.29*	*1.00*	*0.69*
12		S, Sed.	110	40	80	*2.75*	*1.00*	*2.00*
13	A/PC	Intact virus	<40	40	305	<0.13	0.13	*1.00*
14		Ether-tr. virus	<40	40	270	<0.15	0.15	*1.00*
15		B, Sup.	85	<40	<40	>*2.12*	—	—
16		B, Sed.	70	<40	40	*1.75*	<1.00	1.00
17		S, Sup.	60	<40	150	*0.40*	<0.27	*1.00*
18		S, Sed.	80	40	310	*0.26*	0.13	*1.00*

[a] Italic values pertain to reactions where ACU_{AN} titers against NA were found
[b] <40/<40 was scored as —

Table 4. *Coefficients of correlation (r) and linear regression (a′ and b′) for relationship between d_{50} and NIT titers*

y	x	r	a′	b′
Log d_{50} Bel-Sing	Log NIT Bel-Sing	0.8879	0.2146	0.9668
Log d_{50} X 15-HK	Log NIT X 15-HK	0.9092	1.3300	0.9827
Log d_{50} eq.-PC	Log NIT eq.-PC	0.9469	0.5436	0.8455

iii. The coefficients of the correlations (r) and of linear regression ($y = a'x + b'$) were calculated for the relationship of the logarithms of d_{50} values and the logarithms of NIT titers. The results obtaines are charted in Table 4.

Note that for each recombinant a significant correlation ($P < 0.05$) and slopes of the regression lines not significantly differing from 1.0 ($P < 0.05$) were obtained. Furthermore, the intercepts of these regression lines indicate that the photometric ACU test was more sensitive for AN antibody titration than was the NIT.

The difference in sensitivity was 1.64 to 3.5 fold for the recombinants Bel-Sing and eq.-PC and 21 fold for the recombinant X 15-HK. Due to these differences in sensitivity, the d_{50} values against X 15-HK were in some experiments (e.g. Exper. 12) higher than the d_{50} values against the other recombinants, while the reverse was true for the NIT titers.

Identification of AN Antibodies Against Bel-Sing in A/PC Subunit Antisera by Means of Adsorption Experiments

Antisera produced by vaccination with A/PC subunits and, as controls, sera raised by vaccination with intact A/Sing and A/PC virus were adsorbed with an excess of the recombinants Bel-Sing or eq.-PC. The ACU_{AN} titers found in the supernatants and in the unadsorbed sera are listed in Table 5.

Table 5. *Adsorption of antisera with recombinants Bel-Sing and eq.-PC*

1	2	3	4	5	6	7	8	9
			ACU_{AN}-titers against Bel-Sing of sera			ACU_{AN}-titers against eq.-PC of sera		
	Sera raised by vaccination with			adsorbed with			adsorbed with	
Exp. No.	Virus	Preparation	unad-sorbed	Bel-Sing	eq.-PC	unad-sorbed	Bel-Sing	eq.-PC
1	A/Sing	Intact virus	639	0	394	0	0	0
13	A/PC	Intact virus	0	0	0	567	280	0
15	A/PC	B, Sup.	88	0	27.4	0	0	0
16	A/PC	B, Sed.	52	0	20.2	0	0	0
17	A/PC	S, Sup.	23	0	19.7	180	71	0
18	A/PC	S, Sed.	46	0	23.7	380	170	0

Note that adsorption discriminated between AN antibodies against Bel-Sing and eq.-PC, since homologous adsorption of these control sera resulted in complete and heterologous adsorption in incomplete removal of ACU_{AN} titers. The ACU_{AN} titers against Bel-Sing of A/PC subunits antisera were completely adsorbed with Bel-Sing and incompletely adsorbed with eq.-PC. Thus, these sera showed the adsorption patterns expected for the presence of AN antibodies against Bel-Sing.

Discussion

The data presented in this paper warrant the conclusion that vaccination of chickens with subunits produced by treatment of virus with bromelain and Sarkosyl can result in ACU_{AN} titers against both homologous and heterologous NA's. By contrast, vaccination with intact and ether-treated virus yielded ACU_{AN} titers against homologous AN, only.

In many experiments, the sera produced by vaccination with bromelain- and Sarkosyl-treated viruses reacted in the ACU test, in the NIT and in adsorption experiments like sera raised by vaccination with mixtures of the respective viruses.

This finding is interpreted to suggest that the NA's of the strains tested contained antigenic determinants which were not detectable in intact or ether-treated virus:

The NA's of A/Sing and of A/PC were found to share antigenic determinants and the NA of A/HK to share determinants with the NA's of both A/Sing and A/PC.

The failure of ether-treated virus to induce ACU_{AN} titers against heterologous NA could be interpreted as follows: Ether-treatment of influenza virus is known to result in subunits carrying both HA and NA (23). By contrast, treatment of virus with bromelain and Sarkosyl results in at least partial separation of HA and NA (2, 21). Therefore, these findings suggest that separation of both antigens is a prerequisite for unmasking NA antigenic determinants possibly hidden or sterically hindered by adjacent HA.

The finding that vaccination with bromelain- or Sarkosyl-treated virus gave ACU_{AN} titers against heterologous NA's could be explained by means of following two hypotheses:

i. Influenza virus HA's and NA's have been reported to contain strain specific antigens (SSA) and common antigenic components (CAC) (16, 24).

It has been previously found that CAC and SSA antibodies are antibodies of the same specificity but with differences in quality (in terms of their equilibrium constant K for virus-antibody interaction), the CAC antibodies being higher in quality (13).

Then, the conclusion would be reached that vaccination with subunits produced by means of bromelain and Sarkosyl yielded antibodies of higher quality (CAC antibodies) than did vaccination with intact or ether-treated virus. However, since the CAC antibodies would be expected to react both with homologous and the respective heterologous NA, this assumption could not explain the finding that heterologous and no homologous antibody response has been observed in some experiments.

ii. As alternative, it could be assumed that NA's contain hidden antigenic determinants which are present as "major antigen" on the NA of heterologous strains and are only released after splitting of virus.

This interpretation could account for the experimental results described.

At present, no statement can be made, which of theses interpretations is the correct one. Experiments designed to elucidate this question are in progress.

The homologous AN antibody response to vaccination with subunits by treatment with bromelain and Sarkosyl was lower than that obtained with comparable doses of intact or ether-treated virus. Reduced antigenicity of subunit vaccines has been described previously (18) for influenza vaccines obtained by treatment of virus with tri-N-butyl phosphate and Tween 80-ether.

Vaccination with the supernatants of Sarkosyl-treated virus gave in most experiments lower AN antibody response than found for vaccination with the corresponding sediment fractions.

However, since such preparations could contain unknown amounts of NA which was still antigenic but no longer detectable in the neuraminidase test, no conclusions as to the relative antigenicity of these preparations are possible.

The finding that subunits can induce heterologous NA antibody response is of the following practical consequences:

It can be expected that vaccination with subunit preparations could yield a broader AN antibody response than does vaccination with the corresponding intact virus. However, whether or not such broader AN antibody response will also result in broader immunity remains to be tested.

Furthermore, the antigenic heterogeneity of subunit preparations should be taken into account when interpreting the results of serologic tests obtained by use of such preparations (19).

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