Detection of Cells Producing Surface Antigen-Specific Antibody to Influenza Viruses

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B cells producing antibodies to influenza virus antigens were detected and quantitated by a hemolytic plaque assay. Responses of mice after primary infection and immunization with influenza viruses were measured and compared with responses after secondary immunization. The B-cell responses were specific and differentiated between A and B influenza viruses and between different subtypes of A influenza viruses. Responses to closely related influenza A virus strains of the H3N2 subtype cross-reacted but could also be differentiated. Cells secreting antibody to either of the virus surface antigens (hemagglutinin and neuraminidase) could be separately enumerated. Evidence that immunoglobulin G-secreting cells are detected in the assay without the use of facilitating anti-immunoglobulin G sera is presented.

Techniques of preparing pure influenza virus antigens of varying size and complexity are being developed. Influenza viruses, both whole and disrupted, are used in human immunization programs, and it is important to study the processes by which immunization with such antigens leads to production of specific antibodies by B cells. The methods currently used to study the result of immunization with influenza viruses depend on the detection of antibodies in the serum or secretions of the immunized host. This is a relatively crude approach compared with techniques used to study immune responses to simpler chemical antigens. The study of the responses to such antigens has been greatly facilitated by use of the hemolytic plaque assay of Jerne et al. (7). In this report, we present results obtained with a hemolytic plaque assay method which permits differentiation of antibody responses to the strain-specific surface antigens of the virus. In addition, by the use of recombinant viruses, it is shown that antibody responses to the two surface antigens can be separately measured by this assay. This technique advances the study of immune responses to influenza virus antigens beyond the limits imposed by serological tests and provides a basis for quantitative *in vitro* studies on the factors involved in specific B-cell responses to these virus antigens.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from the National Institutes of Health Small Animal Section. Animals of different ages were used in various experiments; mice 6 to 8 weeks old were used for primary immunization, whereas animals for second-

ary-response studies were 14 to 20 weeks old at the time of the secondary immunization.

Viruses. Influenza viruses A/Port Chalmers/1/73 (H3N2) (A/Pt Ch/73), A/Victoria/3/75 (H3N2) (A/Vic/75), A/New Jersey/8/76 (HswN1) (A/NJ/76), and B/Hong Kong/5/75 (B/HK/75) were obtained from virus pools maintained at the Bureau of Biologics and were grown in embryonated chicken eggs by standard procedures. Formalin-inactivated, zonally purified preparations of these virus strains were obtained from influenza vaccine concentrates supplied by a number of vaccine manufacturers. A/Equi-1/Detroit/3/64 (Heq1Neq1) (A/Equi/64) and recombinants between this virus and A/Georgia/101/74 (H3N2), an A/Pt Ch/73-like virus, were kindly supplied by B. Murphy (National Institute of Allergy and Infectious Diseases, Bethesda, Md.); these recombinants had H3Neq1 and Heq1N2 surface antigens. The latter three viruses were grown by standard procedures, and concentrates were prepared by pelleting the virus at $100,000 \times g$ followed by centrifugation through a sucrose gradient. The gradient-purified virus was dialyzed against several changes of phosphate-buffered saline, pH 7.2, before storage at 4°C.

Antibody titrations. Hemagglutination-inhibiting (HI) antibodies in mouse sera were titrated in a standard microassay after removal of nonspecific inhibitors by treatment with receptor-destroying enzyme from *Vibrio comma* (Microbiological Associates, Bethesda, Md.) (12).

Measurement of virus titers in lungs. Infectivity titers of lung homogenates were determined by titration in eggs. End points were calculated by the method of Spearman-Kärber (3) and expressed as 50% egg-infective dose (EID₅₀)/0.2 ml.

Preparation of spleen cell suspensions. Mice were killed by cervical dislocation, and their spleens were removed and placed in 2 ml of Hanks balanced salt solution at 4°C. Spleens were gently disrupted by repeatedly aspirating and expelling the spleen suspen-

sion from a 1-ml syringe, and debris was removed by aspiration. Viability counts using trypan blue were performed on samples of the cell suspensions after lysis of erythrocytes in ammonium chloride buffer (13). Viability was usually 90% or more, with viable cell counts ranging from 25×10^6 to 100×10^6 /ml. Dilutions of the suspension were prepared for specimens likely to contain large numbers of antibody-producing cells.

Preparation of virus-treated erythrocytes.

Target erythrocytes were prepared as described by Russell et al. (14), using 3-week-old sheep erythrocytes (SRBC) from a single animal. Satisfactory results were obtained with SRBC 2 to 4 weeks old; older cells showed extensive lysis during the sensitization procedure. Erythrocytes from two sheep were tested; both gave similar results. Briefly, SRBC were washed three times in Veronal-buffered saline, pH 7.2 (VBS), and pelleted at $500 \times g$. The packed-cell volume was measured, and the SRBC were treated with 1 volume of 5×10^{-4} M KIO_4 in VBS and 10 volumes of various dilutions of influenza virus in VBS. After adsorption of virus for 10 min at room temperature, the cells were washed and resuspended in an equal volume of VBS. Agglutination of SRBC with high concentrations of virus occurred during sensitization of the SRBC, but any clumps of cells were readily dispersed. Satisfactory results were obtained with SRBC sensitized with virus 3 to 4 days previously; longer storage often resulted in nonspecific hemolysis of the SRBC.

Hemolytic plaque assay. A modified version of a thin-layer method described by Jerne et al. (7) was used, since initial experiments indicated that optimal resolution of plaques was obtained with very thin layers of agarose. Spleen cells (0.1 ml of cell suspension) and treated SRBC (0.2 ml of 50% suspension) were added to 2 ml of nutrient agarose at 46°C [0.9% agarose in Eagle minimum essential medium with 1 mg of bovine serum albumin per ml, buffered to pH 7.4 with 0.1 M tris(hydroxymethyl)aminomethane (Tris) and containing 100 U of penicillin and 100 μg of streptomycin per ml]. After gentle mixing, the agarose-cell mixture was poured evenly into a prewarmed (46°C) 100-mm tissue culture-grade petri dish. The

dish was then spun with the fingertips, and the excess agarose at the periphery of the dish was discarded. The remaining agarose was allowed to solidify in a thin layer, and the cultures were then incubated. In each experiment the discarded agarose-cell mixture from a number of plates was weighed, and a correction was made for this loss (usually 40 to 50%) when calculating the number of lymphocytes remaining in each dish.

The direct assay for the detection of plaque-forming cells (PFC) was performed by incubating at 37°C in a humidified atmosphere of 5% CO_2 in air. After 90 min, 3 ml of a 1:10 dilution of guinea pig complement, in Tris-buffered saline, pH 7.4, was layered over the agarose. The complement was removed after additional incubation at 37°C for 30 min. The indirect plaque assay to enhance the detection of immunoglobulin G (IgG) PFC was carried out on duplicate cultures by adding 3 ml of a suitable dilution in Tris-buffered saline of rabbit anti-mouse IgG serum (heavy- and light-chain specific; Miles Research Products, Elkhart, Ind.) during the last 30 min of the first incubation period, followed by complement as described above. Plaques were counted by using a dissecting microscope, and the number of PFC per 10^6 viable retained spleen cells was calculated.

RESULTS

Effect of virus concentration on SRBC. SRBC sensitized with a range of dilutions of A/Pt Ch/73 vaccine were used in direct plaque assays with spleen cells from mice immunized with 100 chick cell-agglutinating units (CCA) of the vaccine 5 days previously. Well-defined plaques approximately 1 mm in diameter were obtained with SRBC sensitized with vaccine diluted to a hemagglutinin HA titer of 1:500 (HA titration with 0.2-ml volumes of virus and 0.5% chick RBC). With higher virus concentration, plaque size was diminished; with lower virus concentrations, plaques were larger and more hazy (Fig. 1). Low plaque counts were obtained

FIG. 1. Influence of SRBC-sensitizing virus concentration on plaque morphology. SRBC sensitized with A/Pt Ch/1/73 virus with HA titer of (A) 1:1,600; (B) 1:500; (C) 1:85.

with SRBC sensitized with nonoptimal viral concentrations. With other virus vaccines, optimal plaque formation was usually produced with similar concentrations of virus (HA titer, 1:500-1:1,000) in tests with spleen cells from mice immunized with the homologous virus.

Virus particle counts (kindly performed by R. Dunlap) were used to determine the number of A/Pt Ch/73 virus particles attached to sensitized SRBC. Ten milliliters of virus suspension containing 1.07×10^{10} particles/ml produced optimal sensitization of 1 ml of packed SRBC (2×10^{10} cells/ml). Thus, five virus particles were bound per erythrocytes. Less well-defined plaques were produced with one-third of this concentration of virus, i.e., 1.7 virus particles per erythrocyte. Efficiency of binding was >99.5%, since no detectable elution of virus from SRBC occurred after adsorption and no hemagglutinating activity was detected in sequential washings of virus-treated SRBC. These calculations assume an equal distribution of virus throughout the SRBC population.

Temporal splenic PFC response of mice to infection and immunization with A/Pt Ch/73 virus. To determine the relative rates of splenic B-cell responses to infection and immunization, four groups of mice were tested in a series of experiments. The groups were: (i) primary intranasal infection with approximately 10^5 EID₅₀ of A/Pt Ch/73 virus under light ether anesthesia, (ii) primary immunization (intraperitoneally [i.p.] with 100 CCA of A/Pt Ch/73 inactivated whole-virus vaccine, (iii) secondary immunization (i.p.) with 100 CCA of A/Pt Ch/73 vaccine given 4 weeks after aerosol infection with A/Pt Ch/73 virus (the aerosol route was used because it is the natural route of infection and induces an immune response to all components of the replicating virus and; (iv) untreated control mice. All animals were female BALB/c mice of the same age. At frequent intervals, five animals per group were killed by exsanguination under ether anesthesia; sera were collected for antibody measurements; then lungs were removed for virus infectivity titrations and spleens were removed for direct and indirect plaque assays against A/Pt Ch/73-treated SRBC. All four groups were tested in each experiment.

The results (Fig. 2) show the mean $\pm$ standard error of the mean for five animals at each point. Specimens from normal mice gave consistently negative results for virus isolation, for serum HI antibody, and for splenic PFC responses and therefore are not presented.

Response to infection. High titers of virus ($10^{6.4}$ EID₅₀/0.2 ml) were recovered from the lungs of infected animals 24 h after infection, but these titers fell rapidly after day 5, with no

FIG. 2. Splenic PFC responses of BALB/c mice to intranasal infection or i.p. immunization with A/Pt Chalmers/1/73 influenza virus. Symbols: (●) Lung virus titer; (▲) serum HI antibody titer; (●) direct PFC/10⁶ cells; (◆) indirect PFC/10⁶ cells. The results show the mean $\pm$ standard error of the mean for five animals at each point. Specimens from normal mice gave consistently negative results for virus isolation, for serum HI antibody, and for splenic PFC responses and therefore are not presented.

detectable virus present by day 10. Serum HI antibody was first detected on day 14 and rose progressively until day 24, when the experiment was terminated. Splenic PFC responses were very low, with a mean direct PFC response of only 5 PFC per 10⁶ cells on day 10 and a mean indirect PFC response of only 3 PFC per 10⁶ cells on day 17.

Response to primary parenteral immunization. Serum HI antibody to A/Pt Ch/73 virus was detected in two of five sera collected 4 days after i.p. immunization, and titers in-

creased to a peak on day 14. Direct PFC were first detected on day 4 (140 per 10^6 cells), with similar numbers on day 5. Thereafter direct PFC numbers declined, with few detected on day 14. The appearance of indirect PFC followed a different time course, appearing 1 day later and peaking at day 7. Few indirect PFC were detected on day 14, and none were present in spleens collected on day 24.

Response to secondary parenteral immunization. After the booster i.p. immunization, serum HI antibody titers rose rapidly and increased 32-fold between days 1 and 10. Both direct and indirect PFC appeared earlier than in a primary response and were detected on day 3, with peak numbers occurring on days 4 and 5. The numbers of direct and indirect PFC did not differ significantly from each other during the period of maximum response. Results presented in detail below suggest that during a secondary response, IgG-producing PFC might be measured by the direct plaque assay.

Virus strain specificity of PFC response. Influenza A virus strains can be distinguished serologically according to the antigenic types of their two surface glycoproteins. To determine whether differences between virus strains possessing different HA and neuraminidase (NA) antigens could be detected in the hemolytic plaque assay, mice were infected intranasally with different strains of influenza A or B viruses and given a booster inoculation (i.p.) of 10 to 100 CCA of homologous inactivated virus vaccine 6 to 8 weeks later. Five days later, spleens were removed from three mice per group and used in a direct plaque assay with SRBC treated with optimal dilutions of virus vaccines. The results showed significant cross-reactions only between A/Pt Ch/73-immune cells and SRBC sensitized

with the closely related A/Vic/75 (H3N2) virus (Table 1). Spleen cells from normal mice had very low background numbers of PFC to all of the target SRBC.

The cross-reaction between the two H3N2 virus strains was examined further (Table 1). Although a high degree of cross-reaction was found, the homologous and heterologous responses were significantly different. Sera collected 8 days after secondary immunization of similar mice with these viruses showed a high degree of similarity between the viruses, with twofold or less differences in HI titer when tested against A/Pt Ch/73 and A/Vic/75 antigens (results not shown).

HA and NA specificity of PFC response. The results presented above indicated that the plaque assay could distinguish between PFC responses to influenza A viruses which differed from each other in both HA and NA antigens. To determine whether this differentiation was based on separate recognition of HA and NA antigens, mice were infected with either A/Pt Ch/73 (H3N2) or A/Equi/64 (Hq1Neq1) viruses followed by booster inoculations i.p. of zonally purified preparations of the corresponding viruses 6 weeks or later after the primary infection. Spleens were removed 5 days later from three or four animals per group and tested individually against SRBC sensitized with the concentrated homologous virus preparations or with recombinant viruses. Spleens from all groups of animals were tested in the same experiment.

Results of a typical experiment (Table 2) show the level of heterologous cross-reactions as percentages of the number of PFC per 10^6 cells observed in the homologous system. Spleen cells from mice immunized with the parent A/Equi/

TABLE 1. *Virus strain specificity of hemolytic plaque assay*

Immune spleen cells ^a	SRBC treated with (direct PFC/ 10^6 cells):				
	A/Pt Ch/73 H3N2	A/Vic/75 H3N2	A/NJ/76 Hsw1N1	B/HK/75	NIL
A/Pt Ch/73	162 ± 73	19 ± 10	0.3 ± 0.3	0	0
A/NJ/76	0.5 ± 0.5	1.0 ± 1.0	137 ± 16	0.5 ± 0.5	1.0 ± 1.0
B/HK/75	0.3 ± 0.3	0.3 ± 0.3	0	51 ± 13	0.3 ± 0.3
Control	0.3 ± 0.3	1.3 ± 0.9	0.7 ± 0.7	0.7 ± 0.7	0
A/Pt Ch/73	178 ± 51	92 ± 37	NT ^b	NT	0
$P = <0.05^c$					
A/Vic/75	47 ± 29	230 ± 81	NT	NT	0

^a Spleens removed 5 days after secondary immunization (i.p.); mean of three or four spleens ± standard error of the mean.

^b NT, Not tested.

^c Student *t* test.

64 (Heq1Neq1) and A/Pt Ch/73 (H3N2) viruses showed no significant cross-reaction with SRBC treated with the heterologous parent virus possessing different HA and NA antigens. In each case these spleen cells, however, demonstrated a good response against SRBC treated with recombinant viruses possessing the homologous HA but heterologous NA antigen, with PFC counts equal to 49 and 36% of those obtained with completely homologous virus (Table 2). A/Pt Ch/73 (H3N2)-immune spleen cells also showed a 50% cross-reaction with SRBC treated with the Heq1N2 recombinant which has the same NA but different HA. A/Equi/64 (Heq1Neq1)-immune spleen cells repeatedly gave low levels of cross-reaction in the system bearing the homologous NA antigen but different HA, although sera from these mice had high antibody titers to Neq1 antigen in NA inhibition assays. These results indicate that PFC responses to both the HA and NA antigens can be separately detected in the hemolytic plaque assay.

Comparison of direct and indirect assays. The primary and secondary splenic PFC re-

sponses 4 or 5 days after i.p. inoculation with 100 CCA of formalin-inactivated A/Pt Ch/73 vaccine were examined in direct and indirect assays. These time points were optimal for demonstrating the switch over from IgM to IgG synthesis in a primary response.

The results from mice receiving a primary immunization showed an increase in number of direct PFC from 7.9 per 10^6 cells on day 4 to 76.1 on day 5, whereas animals undergoing a secondary response had direct PFC counts of 19 and 266 per 10^6 cells on the corresponding days (Table 3). The anti-IgG serum produced a total suppression of plaques in cell preparations taken 4 days after a primary immunization and a partial (but not statistically significant) suppression of plaques from 5-day primary-response cells. This suppression of early direct, presumably IgM, plaques by anti-IgG serum was observed with different lots of rabbit anti-mouse IgG sera but not with normal rabbit serum. Similar suppression of IgM-secreting PFC has been previously reported by other workers using anti-IgG sera (18) and may be due to anti-light-chain activity present in the anti-IgG sera. Spleen cells

TABLE 2. HA and NA specificity of PFC response

Immune spleen cells ^a	Direct PFC response (%) ^b in SRBC treated with:				PFC/ 10^6 cells for homologous assay systems
	A/Pt Ch/73 H3N2	Recombinant Heq1N2	Recombinant H3Neq1	A/Equi/64 Heq1Neq1	
A/Pt Ch/73	100	50 ± 29	49 ± 31	1.7 ± 0.9	601
A/Equi/64	0	36 ± 21	8.6 ± 5.1	100	141

^a Five days after secondary immunization i.p. with zonally purified virus.

^b (Number of PFC against heterologous SRBC)/(number of PFC against homologous SRBC) × 100; mean of three or four spleens ± standard deviation.

TABLE 3. Primary and secondary PFC responses to A/Pt Ch/73 virus

Immunization	Day	PFC/ 10^6 cells			
		Direct	Indirect		Normal rabbit serum
			Anti-IgG (R156) ^a	Anti-IgG (RM6) ^a	
Primary ^b	4	7.9 ± 2.3 ^d	0.1 ± 0.1	1.0 ± 0.2	6.9 ± 4.3
	5	76.1 ± 20.8	33.1 ± 18.9	31.0 ± 6.4	102 ± 17.9
			$P = 0.03$		
Secondary ^c	4	18.8 ± 14.7	57.0 ± 33.6	64.0 ± 37.1	25.8 ± 13.8
	5	266 ± 110	338 ± 82	269 ± 63	313 ± 89

^a R156 and RM6 are different lots of anti-IgG sera.

^b 100 CCA of A/Pt Ch/73 inoculated i.p.

^c Student *t* test; all other horizontal values do not differ significantly.

^d Mean of four or five spleens ± standard error of the mean.

^e Intranasal infection with A/Pt Ch/73 followed by 100 CCA of A/Pt Ch/73 inoculated i.p. 6 weeks later.

from mice undergoing a secondary response showed a statistically insignificant increase in the number of plaques in plates treated with anti-Ig sera compared with the numbers of PFC detected in the direct assay. These results suggest that the primary response at day 4 consists exclusively of IgM-producing cells which can be completely suppressed by heavy- and light-chain-specific anti-IgG sera. By day 5 both IgM- and IgG-producing cells were present, with IgM predominating, but few if any IgM-secreting PFC were detected in the indirect assay.

The results obtained for the secondary response suggest that some IgG-secreting cells may be detected in the direct assay at this later time. Other experiments supporting this conclusion were done by performing direct assays which were first scored and then followed by anti-IgG facilitation on the same culture plate. No significant increase in plaque count was obtained with the anti-IgG sera (results not shown). Because of this doubt as to the relative contribution of IgG-producing cells to the direct PFC counts, no assignment of antibody class will be given to PFC detected by direct assays. It appears, however, that PFC detected in indirect assays are almost exclusively IgG-producing cells.

DISCUSSION

Although hemolytic plaque assays are widely used in studies on immune responses to a variety of nonreplicating antigens, the technique has found only limited application in studies of the immune response to viruses (8, 9, 11, 17). The B-cell response to influenza virus, for example, has been intensively studied, but the observations are mostly based on measurements of antibody in sera or local secretions. Whereas T-lymphocyte responses to influenza antigens have recently received detailed study (2, 4, 5, 19), the details of antibody responses to influenza virus at the cellular level have been largely neglected. A recent development, however, has involved measurement by radioimmunoassay of antibody synthesized *in vitro* by specific B cells (1, 6).

Use of the hemolytic plaque assay for studies of immune responses to virus antigens involves unique problems. High titers of virus are required, since at least one virus particle per RBC is required for adequate sensitization, and problems in binding of the virus to the RBC may occur. Previous studies with influenza ribonucleoprotein antigen (15), Newcastle disease virus (9), and foot-and-mouth disease virus plus hepatitis B antigen (8, 17) used carbodiimide, tannic acid, and chromic chloride, respectively, as coupling agents. The method described here uses high-titered, zonally purified whole-influenza vi-

rus vaccines which have a natural affinity for membrane receptors on the RBC from a number of animal species. Because of its special properties, influenza is particularly well suited to be used in the hemolytic plaque assay. With this technique it will be possible to quantitate the induction of immune B cells in *in vitro* immunization studies with whole and subunit vaccines. Such studies may, for example, investigate the requirements for macrophage activation in the response to such vaccines.

High numbers of splenic PFC were found after parenteral immunization of mice with influenza virus vaccine. Low splenic responses were, however, found after infection of mice with the nonadapted A/Pt Ch/73 virus. This low PFC response of infected mice was reflected in serum HI antibody production, which occurred later and reached peak titers more slowly than after parenteral immunization. In contrast, however, has been the finding of high PFC responses in spleens and draining lymph nodes of ferrets infected with nonadapted low-egg-passage A/Pt Ch/73 virus (10a). Whereas in ferrets the PFC response was detectable 3 days before HI antibody was found in sera, the present study did not show an increased sensitivity of the plaque assay compared with serum antibody assays in measuring the early stages of antibody production.

An earlier report on PFC responses of lymphocytes from influenza-infected mice used influenza ribonucleoprotein as the target antigen (15). Since this antigen is common to all influenza A viruses, this approach would not permit differentiation of immune responses to the viral surface antigens, which define the antigenic properties of the virus strains. The results presented here demonstrate that responses to closely related H3N2 viruses can be distinguished by using SRBC treated with whole-virus preparations and that responses to both the surface antigens of influenza virus could be separately measured by using recombinant viruses.

The total suppression of early-appearing plaques in the primary antiviral response suggested that all plaques detectable in indirect assays with these anti-IgG sera were produced only by IgG-secreting cells. Suppression of IgM plaques by anti-IgG sera has been well documented by Wortis et al. (18). Plaque counts from direct and indirect assays carried out on PFC produced during the secondary response to vaccine, however, were usually not significantly different. This indicated that IgG-producing cells produce plaques detectable by the direct assay when SRBC sensitized with whole influenza virus are used as the indicator system. Studies in progress with blocking antisera to IgG and IgM

further confirm this observation, as does the production of direct plaques by a hybridoma line secreting monoclonal IgG antibody specific for A/Pt Ch/73 hemagglutinin (C. McLaren, unpublished observations). The reason for this phenomenon is not certain. Influenza virus is a densely saturated antigen, with approximately 500 to 600 HA and NA spikes closely arranged on its surface (16), each probably possessing more than one antigenic determinant (6, 10). This is in contrast to the simple spatially non-repetitive epitopes frequently used in many plaque assays. Attachment of IgG molecules to closely spaced virus antigens might therefore allow pairing and activation of the complement pathway during the direct assay in the absence of facilitating antisera. These results and the finding that only 1.7 to 5 virus particles per SRBC are required for complement-mediated antibody lysis suggest that the local epitope density may be more important than the total number of epitopes distributed over the RBC surface. This hypothesis could be tested by sensitizing SRBC with disrupted influenza virus preparations; small viral subunits containing only a few HA or NA molecules might not provide a sufficient density of bound IgG molecules to produce direct plaques of the type described above.

Since these studies were initiated, a similar technique for detection of influenza antibody-secreting cells has been independently developed by C. S. Reiss, Mt. Sinai School of Medicine, New York, N.Y. Preliminary reports indicate results similar to those presented both here and previously (C. McLaren and G. E. Grubbs, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E193, p. 113; C. S. Petigrow and J. L. Schulman, Abstr. Annu. Meet. Fed. Am. Soc. Exp. Biol., 1977, Abstr. 4246).

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