

Humans have antibodies capable of recognizing oncoviral glycoproteins: Demonstration that these antibodies are formed in response to cellular modification of glycoproteins rather than as consequence of exposure to virus

(humoral immunity/cell versus virus-specified antigenic determinants)

MARIANO BARBACID*, DANI BOLOGNESI†, AND STUART A. AARONSON*

*Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205; and †Duke University Medical Center, Department of Surgery, Durham, North Carolina 27710

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ABSTRACT There is controversy in the literature concerning the presence in humans of antibodies directed against the envelope glycoproteins of known oncoviruses. In the present report, we show that antibodies capable of precipitating a wide variety of oncoviral glycoproteins can be demonstrated under certain assay conditions. Substances as diverse as normal components of serum, extracts of bacteria, and even nonprotein molecules such as glycogen also shared the oncoviral glycoprotein determinants recognized by normal human sera. It was found that immunoprecipitation of a given viral glycoprotein by human sera was entirely dependent upon the cell in which the virus was grown. Human sera specifically did not recognize glycoproteins purified from oncoviruses grown in human or higher primate cells. These findings not only demonstrate that the antibodies were directed against cellular rather than the virus-coded antigenic determinants but also exclude the possibility that this immune response was elicited as a consequence of oncovirus exposure.

Oncoviruses are etiologically involved in naturally occurring tumors in a number of species (for reviews, see refs. 1-3). The widespread distribution of these viruses among vertebrates has led to intensive efforts to demonstrate evidence of their presence in man. A major approach has been the application of highly sensitive radioimmunologic techniques to the search for evidence of antibodies to viral structural proteins in human sera or the presence of viral antigens in tissues or body fluids in humans at potentially high risk to virus exposure or disease.

Considerable controversy exists in the literature with respect to the prevalence of antibodies to oncoviruses in human sera, detected by using whole virions (4-12) or purified viral components as probes (13-19). Recently, Kurth and Mikschy (20) reported that a large fraction of human sera were capable of precipitating, at relatively high titers, the purified major envelope glycoprotein (gp70) of the woolly monkey type C virus. These workers ascribed discrepancies with previous reports (15) to the influence of variables on the antigen-antibody reactions (21). The present studies were undertaken in an effort to determine the basis for these discrepancies. Our results confirm that the reactivity of human sera against oncoviral glycoproteins is due to antibody (20). However, we show that the antibodies are not directed against the virus-encoded gene product itself but against associated cell-specified antigenic determinants shared widely in nature.

METHODS

Cells and Viruses. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Colorado Serum, Denver, CO). Mammalian oncoviruses, obtained as double-sucrose-gradient-purified preparations, were either grown in our laboratory or provided by Electro-Nucleonics (Rockville, MD), Pfizer Laboratories (Maywood, NJ), or Frederick Cancer Research Center (Frederick, MD) through the Office of Resources and Logistics, National Cancer Institute.

Oncoviral Glycoproteins. The major envelope glycoproteins of several oncoviruses were isolated to apparent homogeneity (as determined by NaDodSO₄/polyacrylamide gel electrophoresis) by a combination of ion exchange, gel filtration, and affinity chromatographic techniques. They include: Rauscher murine leukemia virus (MuLV) gp70 (22); Moloney MuLV gp70 (23); AKR MuLV gp70 (24); BALB-virus-2, NIH-MuLV, and NZB-MuLV gp70s (25); feline leukemia virus (FeLV) gp70 (26); simian sarcoma-associated virus (SSAV) gp70 (27); RD114, baboon (*Papio cynocephalus*) endogenous virus (BEV) and Mason-Pfizer monkey virus (MPMV) gp70s (28); and squirrel monkey retrovirus (SMRV) gp90 (29). Purified glycoproteins were labeled to high specific activity with ¹²⁵I by the chloramine-T method (30).

Radioimmunoassays. The experimental conditions described by Kurth *et al.* (7) were utilized throughout the present study. However, for comparative purposes, some assays were performed utilizing our standard radioimmunoassay conditions (25). Protein concentrations were determined as described by Lowry *et al.* (31).

Treatment of Rauscher MuLV gp70 with Glycosidases. ¹²⁵I-Labeled Rauscher MuLV gp70 (5 × 10⁶ cpm) was incubated for 4 hr at 37°C in isotonic phosphate-buffered saline containing Ca²⁺ and Mg²⁺ with 0.3 unit of β-glucosidase, 0.5 unit of α-galactosidase, 1 unit of β-galactosidase, 0.005 unit of β-N-acetylglucosaminidase, 0.3 unit of β-amylase, and 0.02 unit of neuraminidase (P-L Biochemicals). A small aliquot (5 × 10⁴ cpm) was analyzed by NaDodSO₄/polyacrylamide gel electrophoresis to determine the extent of the reaction as monitored by the disappearance of radioactivity from the 70,000-dalton glycoprotein molecule (80-90%). The above enzymes did not exhibit proteolytic activity as determined by the lack of gen-

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Abbreviations: MuLV, murine leukemia virus; FeLV, feline leukemia virus; SSAV, simian sarcoma-associated virus; BEV, baboon endogenous virus; MPMV, Mason-Pfizer monkey virus; SMRV, squirrel monkey retrovirus.

eration of low molecular weight products (<10% of the total input radioactivity) and by the integrity of ^{125}I -labeled ovalbumin and human IgG molecules after treatment under the same experimental conditions.

RESULTS

Comparison of Assay Conditions for Precipitation of Rauscher MuLV gp70 by Human Sera. We set out to determine the basis for the striking differences in results with the assay conditions of Kurth and Mikschy (20) and with those of other laboratories for immunoprecipitation of oncoviral glycoproteins. The ability of normal human sera to precipitate ^{125}I -labeled Rauscher MuLV gp70 was analyzed under both sets of reaction conditions. With our standard assay conditions (25), none of 200 normal sera analyzed exhibited detectable reactivity. With the Kurth and Mikschy assay conditions, approximately 40% of the same sera precipitated more than 50% of the ^{125}I -labeled probe at a 1:10 serum dilution. A similar percentage of sera exhibited limited precipitation (10–50%), whereas only 20% of the samples had no detectable (<10%) immunoprecipitating activity. In contrast, when we analyzed the ability of known antisera against Rauscher MuLV gp70 or related viruses to precipitate the same probe, there was no apparent difference in assay sensitivity.

The effect of each of the individual components of the immunoprecipitation reaction mixtures was next investigated. None of the ionic conditions—including buffers (Tris, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, phosphate), pH (6.5 to 8.0), or cations (Na^+ versus K^+ , presence of Mg^{2+} or Ca^{2+})—seemed to affect the extent of the immunoprecipitation. However, a high concentration of commercially available bovine serum albumin (1%, wt/vol), as routinely utilized in our standard assay (25), completely blocked the immunoprecipitating activity of human sera.

Distribution of the Rauscher MuLV gp70 Antigenic Determinants Recognized by Human Sera. On the basis of the above results, we further investigated the specificity of the reaction by utilizing the conditions of Kurth and Mikschy (20). We first confirmed that the precipitating activity was due to antibody (20). The activity copurified with IgG, and precipitation of the antigen-antibody complexes required antibody specific for human γ heavy chain (data not shown). We next selected a human serum (no. 3975) from a normal blood donor that exhibited a titer of 1:1200 for precipitation of ^{125}I -labeled Rauscher MuLV gp70. A wide variety of reagents were found to displace the radioactive probe when tested as competing antigens in an immunoassay in which limiting amounts of human serum 3975 were used to precipitate ^{125}I -labeled Rauscher MuLV gp70 (Table 1). These included normal sera from species as diverse as chickens, mice, hamsters, rabbits, cows, and New World primates. In contrast, sera from either Old World primates, apes, or humans exhibited no significant competition. Individual bovine serum components such as α -, β -, or gamma globulin fractions were reactive. Thus, it could not be ascertained whether the extensive competition observed with all tested oncoviruses and cell extracts was due to their intrinsic properties or to the presence of contaminant calf serum components present in all biological materials grown in tissue culture. Finally, even such reagents as *Escherichia coli* extracts and glycogen also exhibited significant competition. Similar results were obtained in analogous competition radioimmunoassays developed with each of 100 individual human sera analyzed.

In contrast, when the same antigens were tested in a homologous Rauscher MuLV gp70 assay or in an anti-BALB:

virus-2-vs.- ^{125}I -labeled Rauscher MuLV gp70 group specific immunoassay, only Rauscher MuLV or mouse type C viruses,

Table 1. Distribution of Rauscher MuLV gp70 antigenic determinants recognized by normal human and caprine hyperimmune sera

Competing antigen	% competition in radioimmunoassays* in which R-MuLV gp70 was precipitated by:		
	Human serum 3975	Goat anti-R-MuLV	Goat anti-BALB-V-2
Oncoviruses (0.1 mg/ml):[†]			
Rauscher MuLV	100	100	100
BALB:virus-2	90	30	100
FeLV	95	<10	10
SSAV	95	<10	<10
BEV	90	<10	<10
MPMV	95	<10	<10
SMRV	100	<10	<10
Rous sarcoma virus	85	<10	<10
Avian myeloblastosis virus	50	<10	<10
Cell lines (2 mg/ml):			
Mouse NIH/3T3	95	<10	<10
Rat NRK	95	<10	<10
Canine D17	100	<10	<10
Mink Mv1Lu	95	<10	<10
Human A172-10	90	<10	<10
Human A673	95	<10	<10
Normal serum (15 mg/ml):[‡]			
Chicken (<i>n</i> = 3)	50–70	<10	<10
Mouse (<i>n</i> = 5)	90–95	<10	<10
Rat (<i>n</i> = 8)	95–100	<10	<10
Hamster (<i>n</i> = 10)	95–100	<10	<10
Rabbit (<i>n</i> = 5)	90–95	<10	<10
Bovine (<i>n</i> = 5)	95–100	<10	<10
Fetal calf (<i>n</i> = 2)	95–100	<10	<10
New World primate serum (<i>n</i> = 13):			
Marmoset	95–100		
Capuchin monkey	95–100	<10	<10
Spider monkey	95–100	<10	<10
Owl monkey	95–100	<10	<10
Old World primate serum (<i>n</i> = 22):			
Rhesus	<10	<10	<10
Gibbon	<10	<10	<10
Orangutan	<10	<10	<10
Gorilla	<10	<10	<10
Chimpanzee	<10	<10	<10
Human serum	<10	<10	<10
Serum (bovine) components (2 mg/ml):			
α -Globulin	100	<10	<10
β -Globulin	100	<10	<10
Gamma globulin	85	<10	<10
Other antigens:			
<i>Escherichia coli</i> cell extract (2 mg/ml)	65	<10	<10
Glycogen (10 mg/ml)	70	<10	<10

* Radioimmunoassays, performed as described by Kurth *et al.* (7), included those in which limiting amounts of human serum 3975 or hyperimmune caprine sera elicited against Rauscher MuLV (R-MuLV) or BALB:virus-2 (BALB-V-2) were used to precipitate 10^4 cpm of ^{125}I -labeled Rauscher MuLV gp70.

[†] All oncoviruses were grown in tissue culture conditions except avian myeloblastosis virus which was purified from plasma of infected chickens.

[‡] *n*, Number of samples.

respectively, exhibited significant competition (Table 1). The above results indicated that the antigenic determinants of Rauscher MuLV gp70 recognized by normal human sera under the Kurth and Mikschy assay conditions were not unique to this molecule but were shared very widely in nature.

Interaction of Human Sera with Glycoproteins Isolated from Oncoviruses of Different Origins. To characterize further this serological reaction, we analyzed the ability of human sera to precipitate various oncoviral glycoproteins purified in our laboratory. Table 2 shows the results obtained with 10 representative human serum samples. A well-defined, although surprising, pattern of immunoprecipitation was observed: (i) human sera capable of precipitating Rauscher MuLV gp70 also reacted with Moloney MuLV gp70, NIH-MuLV gp70, FeLV gp70, and SMRV gp90; (ii) their titers were independent of the viral origin of the ¹²⁵I-labeled glycoprotein; (iii) NZB-MuLV gp70, SSAV gp70, BEV gp70, and MPMV gp70 were not recognized by any of the tested sera; and (iv) some samples did not precipitate any of the nine ¹²⁵I-labeled viral glycoproteins tested.

This pattern of oncoviral gp70 precipitation was different from that observed with antisera experimentally elicited against either virions or purified glycoproteins. For instance, anti-mouse type C viral gp70 antiserum recognizes related antigenic determinants in analogous molecules of feline but not endogenous primate oncoviruses. Similarly, antibodies elicited against BEV gp70, MPMV gp70, or SMRV gp90 have not been shown to react with MuLV or FeLV glycoproteins (25, 27-29). Most strikingly, NZB- and NIH-MuLV gp70s, which are indistinguishable by standard radioimmunologic techniques (25), were readily discriminated by human sera: serum samples that precipitated NIH-MuLV gp70 did not appear to recognize NZB-MuLV gp70 under the Kurth and Mikschy assay conditions.

Human Antibodies Interact with Oncoviral Glycoproteins by Recognizing Cellular Rather than Virus-Encoded Antigenic Determinants. From the immunoprecipitation pattern, it became apparent that all nonprecipitable viral glycoproteins were purified from viruses grown in cells of human or higher primate origin. These findings, along with the lack of competition by sera from those same species in the human serum 3975-vs.-¹²⁵I-labeled Rauscher MuLV gp70 assay, suggested that the observed human antibodies might be directed against cell-specified, rather than virus-encoded antigenic determinants associated with the glycoprotein molecule.

To investigate this possibility, we tested various purified oncoviral glycoproteins in the human serum 3975-vs.-¹²⁵I-labeled Rauscher MuLV gp70 competition radioimmunoassay.

Table 3. Competition of purified envelope glycoproteins of mammalian oncoviruses for Rauscher-MuLV gp70 antigenic determinants recognized by normal human sera

Viral glycoprotein	From virus grown in	% competition in radioimmunoassays* in which R-MuLV gp70 was precipitated by:	
		Human serum 3975	Goat anti-BALB: virus-2
		Rauscher MuLV gp70	100
Moloney MuLV gp70	90	100	
AKR MuLV gp70	80	100	
BALB:virus-2 gp70	90	100	
SMRV gp90	80	<10	
NZB-MuLV gp70	<10	100	
RD114 gp70	<10	<10	
BEV gp70	<10	<10	
MPMV gp70	<10	<10	
NIH-MuLV gp70	95	100	
NIH-MuLV gp70	<10	100	
FeLV gp70	80	<10	
FeLV gp70	<10	<10	
SSAV gp70	95	<10	
SSAV gp70	<10	<10	

* Radioimmunoassays included those in which limiting amounts of human serum 3975 or caprine anti-BALB:virus-2 sera were used to precipitate 10⁴ cpm of ¹²⁵I-labeled Rauscher MuLV gp70. Experimental conditions were those described by Kurth *et al.* (7).

Only those glycoproteins purified from viruses grown in cells of nonprimate origin exhibited significant competition (Table 3). This observation was more clearly illustrated when glycoproteins were purified from viruses capable of actively replicating in cells of human and nonhuman origin. Viral gp70s purified from NIH-MuLV, FeLV, and SSAV and grown in canine, kitten lung, and rat cells, respectively, were able to displace the radioactive viral probe extensively. In striking contrast, glycoproteins isolated from the same viruses grown instead in human cells exhibited no detectable competition. Control experiments indicated that the type, group, and interspecies antigenic determinants detected in each of these viral glycoproteins with experimentally obtained hyperimmune sera were independent of the origin of the cell line in which the virus was grown. These results conclusively demonstrated the cellular rather than viral specificity of the antigenic determinants recognized by normal human sera.

Table 2. Precipitation of envelope glycoproteins of different oncoviruses by representative human sera

Human serum	Antiserum titers* for precipitation of ¹²⁵ I-labeled oncoviral glycoproteins purified from								
	R-MuLV†	M-MuLV†	NIH-MuLV	NZB-MuLV	FeLV	SSAV	BEV	MPMV	SMRV
3945	<10	<10	<10	<10	<10	<10	<10	<10	<10
3959	<10	10	<10	<10	<10	<10	<10	<10	<10
3975	1200	960	640	<10	640	<10	<10	<10	960
3980	320	240	320	<10	240	<10	<10	<10	240
3982	240	240	160	<10	120	<10	<10	<10	120
3991	160	240	200	<10	160	<10	<10	<10	160
3994	200	240	240	<10	120	<10	<10	<10	160
4000	80	160	120	<10	60	<10	<10	<10	80
4006	<10	<10	<10	<10	<10	<10	<10	<10	<10
4017	<10	<10	<10	<10	<10	<10	<10	<10	<10

* Titers are expressed as the reciprocal of the highest serum dilution capable of binding 20% of the appropriate ¹²⁵I-labeled retroviral glycoprotein.

† R, Rauscher; M, Moloney.

Table 4. Precipitation of carbohydrate-depleted Rauscher-MuLV gp70 by different antisera

¹²⁵ I-Labeled Rauscher MuLV gp70	Human serum 3975		Anti-Rauscher MuLV		Anti-Rauscher MuLV gp70	
	Titer*	Max. binding†	Titer*	Max. binding†	Titer*	Max. binding†
Untreated	1:800	88	1:1600	95	1:12,500	98
At 37°C for 4 hr	1:800	76	1:1500	86	1:9,500	88
At 37°C for 4 hr in presence of glycosidases	<1:10	8	1:700	50	1:5,000	60

¹²⁵I-Labeled Rauscher MuLV gp70 (5×10^6 cpm) was incubated in the presence or absence of a mixture of glycosidases for 4 hr at 37°C in a 25- μ l reaction mixture containing isotonic phosphate-buffered saline with Mg²⁺ and Ca²⁺. At the end of the incubation period, the sample was diluted at least 1:100 with radioimmunoprecipitation buffer (7) and incubated with 1:2 serial dilutions of the appropriate serum.

* Titers are expressed as the highest serum dilution capable of binding 20% of the ¹²⁵I-labeled Rauscher MuLV gp70.

† Maximum binding values represent the percentage of radioactive glycoprotein bound at the lowest serum dilution tested (1:10).

Kurth and Mikschy (20) have reported that SSAV gp70 purified from virions grown in marmoset cells (32) was readily immunoprecipitated by human sera. In contrast, none of the human sera analyzed in the present study recognized our SSAV gp70, which was purified from virions grown in human NC37 cells. This correlated with our observations that New World primate sera possessed those antigenic determinants associated with glycoproteins of oncoviruses grown in cells of nonhuman origin (Table 1). These findings further argue for the species specificity of the cellular modification of viral glycoproteins which resulted in their immune recognition by human sera.

Table 4 provides some experimental evidence suggesting that the cell-encoded antigenic determinants were mostly localized within the carbohydrate domain of the gp70 molecule. When ¹²⁵I-labeled Rauscher MuLV gp70 was treated with a combination of exoglycosidases, the product of the reaction was no longer precipitated by human sera under the Kurth and Mikschy assay conditions. In contrast, the same treatment only partially affected those antigenic determinants recognized by caprine antisera elicited against Rauscher MuLV virions or purified gp70 glycoprotein. However, conclusive proof that carbohydrate is the sole moiety reactive with the human sera remains to be obtained. We have observed that endoglycosidase-digested glycoproteins demonstrate a striking degree of self-aggregation, making them very difficult to study by radioimmunologic techniques. Moreover, the extent of carbohydrate removal is probably less than total in most of the digestions we have attempted. Thus, although carbohydrate remains the most likely target, we cannot exclude the existence of human antibodies directed against other cell-specified, nonvirus-encoded antigenic determinants present in oncoviral glycoproteins.

DISCUSSION

The demonstration of a humoral immune response in humans to an oncovirus could have considerable implications with respect to the etiologic involvement of such viruses in human cancer. As a consequence, this area of research has been the subject of intensive investigation by a number of laboratories utilizing different approaches. There has been controversy in the literature with respect to results and, in some cases, to their interpretation. Human antibodies have been reported to be capable of reacting with type C virions (4-12). However, care must be exercised in interpreting such data because such activity can be directed against calf serum globulins associated with the virus preparations (4, 11, 12, 33).

With more refined systems in which purified proteins rather than whole viruses have been used as probes, contradictory results have also been reported. Kurth and Mikschy (20) detected a high prevalence of antibodies capable of precipitating the envelope glycoprotein of the woolly monkey type C virus.

However, another study found no evidence of reactivity against this viral protein in a similar survey of human sera (15). Because of the well-characterized nature of the components involved in these immunoprecipitation reactions, we thought that this controversy should be amenable to resolution.

We found that the contradictory results were due to assay conditions. In contrast to a recent report by Kurth *et al.* (21), differences in buffers, pH, or ionic conditions had little or no effect on the ability of human sera to recognize oncoviral glycoproteins. Instead, the concentration of commercial bovine serum albumin utilized as carrier was the critical factor in determining whether a given human serum registered as positive. Comparable amounts of other carrier proteins, such as ovalbumin and lysozyme, or lower amounts of bovine serum albumin (<0.1%) did not block the immunoprecipitating reactivity. These results suggested that bovine serum albumin or, more likely, a contaminant of this commercial product contained the antigenic determinants of Rauscher MuLV gp70 recognized by human sera. Substances as diverse as normal components of serum, extracts of bacteria, and nonprotein molecules such as glycogen also shared these determinants. These results, by themselves, did not resolve whether the humoral immunity was a response to a specific oncoviral protein or to any of the large number of immunologically crossreactive substances.

Despite the wide distribution in nature of substances that shared these antigenic determinants, not all oncoviral glycoproteins possessed them. The presence of these determinants was found to be independent of the known evolutionary relationships of oncoviruses (24-29). We were able to show that viral glycoproteins of oncoviruses grown in human cells were not immunoprecipitated by human sera, indicating that recognition was determined by the cell in which the virus was grown. Thus, if such human antibodies were elicited as a consequence of human exposure to oncoviral glycoprotein rather than any of the large number of other substances possessing related antigenic determinants, this putative virus could not have replicated in the "infected" human host. Moreover, viral glycoproteins of each of three separate viruses grown in rodent or carnivore cells, but not those of the same viruses grown instead in human cells, were recognized by human sera. Therefore, these antibodies must be directed against antigenic determinants specified by the host cell rather than against the viral-encoded gene product. These findings exclude the possibility that this human immune response was elicited as a result of oncovirus exposure.

Our results, as well as those of Snyder and Fleissner (33), indicate that the most likely target of the antibodies is the carbohydrate moiety associated with oncoviral as well as other glycoproteins. However, the possibility that at least part of the humoral response may be directed against other cell-specified antigenic determinants cannot be excluded. It is known that

glycosylation exhibits species specificity (34-37). We also observed a striking pattern of species specificity with respect to the cell in which a virus was grown and the ability of human sera to recognize its envelope glycoprotein. Thus, the ability of humans to develop antibodies to carbohydrate antigenic determinants coded for by other species may confer to any protein, including viral proteins that acquire such determinants, immunologic recognition as well. Whether such antibodies provide a host defense mechanism against transmission to humans of viruses from other species is an interesting possibility that warrants further investigation.

The specificity of the immune response of human sera against purified oncoviral glycoproteins has been analyzed in 100 serum samples. These have included sera from normal persons, tumor patients, laboratory workers, and veterinarians as well as sera kindly provided by R. Kurth. In all cases, the immune reaction was shown to be due to cell-specified rather than virus-encoded antigenic determinants and could not have been elicited as a result of oncovirus infection. In addition, it must be emphasized that, in previous studies in which hundreds of samples from normal and clinically affected humans were tested under conditions that eliminated the possibility of this phenomenon, no antibodies against oncoviruses were detected (15, 18, 19). The fact that humans can respond immunologically to experimentally administered type C viral antigens (38) has, however, been demonstrated (14, 15). Whether genetically transmitted oncoviruses or as yet unknown infectious oncoviruses play a role in human disease remains to be determined. In any event, our studies, as well as those of Snyder and Fleissner (33), raise a note of caution that should be taken into account in future efforts aimed at detection of antibodies to oncoviruses in man.

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