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Antigen-specific nonimmunoglobulin factor that neutralizes xenotropic virus is associated with mouse serum lipoproteins

(murine C-type virus/ultracentrifugal separation)

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ABSTRACT A soluble nonimmunoglobulin factor that specifically neutralizes mouse xenotropic C-type virus is found in normal mouse sera. It is stable from pH 2 to neutrality and resists ether extraction, freezing, or brief heating to 100°. It can be separated from immunoglobulins by ultracentrifugation at a density of 1.21 g/cm^3. Neutralizing activity is only found with the serum lipoproteins in the fraction with density less than 1.21 g/cm^3.

Two major classes of type-C RNA viruses, distinguished by their host range, are endogenous to house mice (1, 2). *Ecotropic* viruses infect mouse cells and produce malignancies in mice (2, 3), whereas *xenotropic* viruses, also integrated in the mouse genome, cannot exogenously infect mouse cells. They productively infect a wide variety of heterologous mammalian and avian cells (1, 2, 4, 5). A disease phenomenon has not been assigned to these viruses.

Mouse sera specifically neutralize xenotropic but not ecotropic viruses (2, 4, 6, 7). We determined that this neutralization was not effected by immunoglobulins, but is attributable to some other soluble serum factor (8). This observation has been confirmed by others (9). Because our evidence has not yet indicated the fate of the virus after interacting with this factor, we have chosen to continue to use the term *virus neutralization*.

Because both xenotropic virus and the soluble neutralizing factor (NF) are present in all house mice, we have proposed that NF plays a role in the regulation of endogenous xenotropic viruses. By interacting with the virus at the cell surface, it may change the internal milieu of cells and thereby affect normal life processes (10, 11). Further characterization of this serum factor is described in this report.

MATERIALS AND METHODS

Materials. Enzymes used in this study came from Sigma Chemical Co. unless otherwise stated. They included pancreatic RNase, DNase-free, heated to 100° for 10 min and stored at −20°; pancreatic DNase, electrophoretically purified, RNase-free (Worthington Biochemicals Corp.); Pronase (*Streptomyces griseus* protease), Calbiochem, self-digested for 2 hr, 37°; trypsin (Tryptar®), Armour Co. crystallized; protease K, (Merck Biochemical Lab.); pepsin, crystallized; amylase (*Aspergillus oryzae*); hyaluronidase type VI (bovine testis); neuraminidase VI (*Clostridium perfringens*); and phospholipase A (wheat germ). All enzymes were assayed for activity prior to their use.

Cells and Viruses. Primary NIH Swiss mouse embryo fibroblasts (NIH-ME) were obtained from Microbiological Assoc., Bethesda, Md., and used for XC plaque assays (12). A clonal line of normal rat kidney cells (NRK) (13) was used for focus formation assays. All cells were maintained in Eagle's minimal essential medium (EMEM) with 10% unheated fetal calf serum, 1% glutamine, and 1% antibiotics (250 units/ml of penicillin; 250 μg/ml of streptomycin). They were treated with DEAE-dextran prior to virus inoculation (14). For focus formation assays, EMEM supplemented with 5% heated (56°, 30 min) calf serum was used.

The NZB pseudotype of the Harvey strain of murine sarcoma virus (MSV), MSV(NZB-MuLV), was obtained from a clone of MSV-transformed NZB mouse embryo cells that produced the NZB xenotropic virus (NZB-MuLV) as well (15). The ecotropic virus, AKR-L1 murine leukemia virus (ARK-MuLV), was originally obtained from J. W. Hartley, Bethesda, Md. and was propagated in NIH-ME fibroblasts in this laboratory.

Virus Assays. Ecotropic MuLV was assayed in NIH-ME by the XC plaque assay and recorded as plaque-forming units/ml. (12). The presence of MSV(NZB-MuLV) was determined by focus formation in normal rat kidney monolayer cells (15) and recorded as focus-forming units/ml.

Sera. Serum was obtained from NZB mice from this laboratory. CBA, C3H, and (CBA × C57/Bl)F$_1$ sera were provided by R. Barnes, London. (B6C3)F$_1$ sera were given to us by J. Ihle, Frederick, Md. Sera were generally heat-inactivated (56°, 30 min) before use although heating did not change the neutralizing activity (8). Dilutions of sera were made in EMEM without calf serum. Antiserum to NZB-MuLV was prepared in rabbits (15).

Neutralization Assays. Initially, mouse sera were titered for NF in serial 10-fold dilutions. More accurate serum titers were subsequently obtained by 2-fold dilutions of that serum dilution which had shown effective neutralization. Because the envelope coat of the MSV(NZB-MuLV) contains the xenotropic virus type-specific antigen, this pseudotypic sarcoma virus can be used effectively in neutralization tests. A reduction in the focus formation induced by this virus in NRK cells was used to quantitate the neutralizing activity against xenotropic virus (8). Reduction in XC plaque formation was used to quantitate neutralizing activity in mouse sera against ecotropic virus. For the neutralization assays, 0.1 ml of each dilution of mouse serum was incubated with an equal amount of MSV(NZB-MuLV) (20,000 focus-forming units/ml) or AKR-MuLV (20,000 plaque-forming units/ml). After 30 min at room temperature, the virus–serum mixture was diluted 10-fold in EMEM to a final concentration of approximately 200 focus-forming units/ml. A 0.4 ml aliquot of this preparation was adsorbed to NRK or NIH-ME for 30 min at 37°. Control cultures received virus incubated with EMEM. The NRK cultures were then fed with EMEM containing 5% heated calf serum. The NIH-ME cul-

Abbreviations: NF, neutralizing factor; MSV, murine sarcoma virus; MuLV, murine leukemia virus; EMEM, Eagle's minimal essential medium.

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tures received EMEM with 10% unheated fetal calf serum. The titer of neutralization was expressed as the reciprocal of the highest serum dilution that reduced by 67% the number of foci or plaques formed in control cultures. The effect of physical, chemical, and enzymatic treatments on NF was determined by assaying the titer of neutralizing activity remaining in mouse serum.

Treatment with Acid and Base. The pH of samples of serum was adjusted to 2 or 12 by adding 1 M HCl or 1 M NaOH, respectively. They were incubated for 15 hr at 4°, and then neutralized to pH 7.0. Precipitates that formed during these treatments redissolved upon neutralization. Sera were also mixed with perchloric acid ($HClO_4$) to a concentration of 2% and held at 0° for 30 min, during which time a precipitate formed. 0.72 M KOH and 0.6 M $KHCO_3$ were then added and the mixture was kept at 0° for 5 min. The precipitate redissolved and the sample was dialyzed for 2 hr in 0.1 M phosphate-buffered saline (pH 7.0) without calcium and magnesium.

Extraction with Ether, Ethanol/Ether, and Chloroform/Methanol. Mouse serum or EMEM containing fetal calf serum was extracted with an equal volume of buffer-saturated, peroxide-free, diethyl ether for 10 min at 0° with shaking. After removal of ether, the aqueous phase was diluted 10-fold in EMEM and assayed for neutralizing activity. Mouse serum was extracted with 20 volumes of ethanol/ether 3:1 at 4° for 15 hr with intermittent shaking. The precipitate was collected by centrifugation (4000 *g*·min) and washed with ether. Remaining ether was removed by evaporation and the precipitate was resuspended in EMEM. The solvent phase was evaporated to dryness and the residue was resuspended by sonication in EMEM for assay. Mouse serum was also extracted for 20 hr with 20 volumes of chloroform/methanol (2:1) at 25°. After the addition of water, the chloroform phase was dried under N_2 and its solids were dispersed in EMEM by sonication.

Treatment with Enzymes. A 10-fold dilution of mouse serum, 0.1 ml, was mixed with 0.1 ml of each enzyme preparation. After a 1- to 2-hr incubation at 37°, the mixture was diluted 10- and 100-fold and assayed for residual neutralizing activity. Pepsin digestion was performed in 0.01 M HCl and the serum sample was then neutralized with NaOH. Phospholipase treatment was conducted in the presence of $CaCl_2$ (6 mM). Controls for these experiments contained the enzyme and the appropriate enzyme diluent. They were treated in the same manner as the experimental samples and tested for any direct inactivation of MSV(NZB-MuLV).

Sucrose Density-Gradient Centrifugation. Density gradient ultracentrifugation was performed according to Leong *et al.* (16). Sucrose concentrations in the region of the serum neutralizing activity had no effect on virus infectivity. Fractions containing activity were pooled, concentrated by rotary evaporation, and dialyzed against 0.01 M Tris·HCl, pH 7.4.

Polyacrylamide Gel Electrophoresis. A 1.5 mm thick slab of polyacrylamide, consisting of stacking (2.0 cm high, 4.5% acrylamide) and separating (12 cm high, 10% acrylamide) gels, was cast as described by O'Farrell (17). The sample was concentrated 10-fold by evaporation, dissolved in 10% (vol/vol) glycerol, 5% 2-mercaptoethanol, 2.3% sodium dodecyl sulfate, 0.0625 M Tris·HCl at pH 8.3, and loaded into wells in the gel. After electrophoresis for 4–6 hr at 6 mA, the gel was cut into horizontal sections which were homogenized in 5 ml of phosphate-buffered saline without calcium and magnesium at 4°. Gel fragments were removed by centrifugation (150,000 *g*·min, 4°) and the supernatants were assayed for NF.

Gel Permeation Chromatography and Preparative Ultracentrifugation of NF. Unheated $(B6C3)F_1$ mouse serum was separated on a 1.2 × 100 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) using 0.15 M NaCl containing 0.5 mM EDTA, pH 8.6. Eluate fractions were analyzed for protein (18), lipid phosphorus (19), cholesterol and triglyceride (20), and were examined for lipoproteins using agarose gel electrophoresis (21). IgG, IgA, and IgM were located in the eluate by immunodiffusion using univalent antibodies (Tago, Inc., Burlingame, Calif.). Mouse lipoproteins were isolated from sera by preparative ultracentrifugation at a density of 1.21 g/cm^3 using a modification of the method of Havel *et al.* (22). The nonprotein solvent density of serum was adjusted to 1.21 g/cm^3 with solid KBr. The serum was overlaid with a solution of KBr/NaCl of density 1.21 g/cm^3 containing 0.05% sodium azide and 1 mM EDTA, pH 8.0, and centrifuged for 24 hr at 105,000 × *g*, at 12° in the 40.3 rotor in a Beckman L3-40 ultracentrifuge. At this time two layers were readily recognized. The supernatant lipoprotein fraction was removed by slicing the tube through the clear intermediate zone with a slicing device. The lipoprotein fraction was purified by recentrifugation after overlayering with 4 volumes of density d = 1.21 g/cm^3 salt solution. The final supernatant fraction (d < 1.21 g/cm^3) and the initial infranatant fraction (d > 1.21 g/cm^3) were dialyzed against 0.15 M NaCl containing 0.5 mM EDTA at pH 7.0. Agarose gel electrophoresis was employed to examine the content of lipoproteins and non-lipid-bearing proteins in the d < 1.21 fraction (21). The lipoprotein fraction was examined for mouse IgM, IgA, IgG, and albumin by immunodiffusion using specific antibodies.

RESULTS

Neutralizing activity of mouse sera: Effect of treatment with anti-NZB-MuLV antiserum

All the mouse sera used in this study significantly neutralized xenotropic viruses but not ecotropic viruses. The NF titer in the sera remained constant for several months at 4°, −20°, and −70° and enabled the frequent use of these sera for characterization of the factor.

To rule out the possibility that NF was a viral envelope component, mouse serum diluted 10- and 100-fold was incubated with rabbit anti-NZB-MuLV antiserum. After 30 min at 25°, the mixture was diluted 10- and 100-fold past the effective neutralization titer of the rabbit antiserum. These dilutions did not exceed the titer of antixenotropic virus neutralizing activity in the mouse sera. The mixture was centrifuged at 1.5 × 10^5 *g*·min at 4° and the supernatant was assayed for NF activity against the NZB pseudotype MSV. The rabbit antiserum had no effect on the titer of NF in mouse sera (Table 1).

Physical-chemical characteristics of xenotropic virus neutralizing factor

Heating and pH. The factor was stable to lyophilization and to dialysis against EMEM at 4° for 2 days. Activity was fully recoverable after heating at 100° for 10 min. NF persisted to a somewhat lesser extent after 20 and 30 min at 100° (Table 1). Heating for 60 min at 56° did not affect NF in mouse sera.

Neutralizing activity remained in the sera after acid treatment, but not after the pH of the serum was raised to 12 (Table 1). Control samples containing only EMEM or EMEM with fetal calf serum, treated in a similar manner, had no effect on focus formation by the virus.

Solvent Extractions. Ether extraction of mouse serum did not affect its neutralizing activity (Table 1), which remained in the aqueous phase. EMEM alone treated similarly did not

Table 1. Properties of neutralizing factor present in mouse sera

Treatment	Neutralizing titer	
	Control*	Sample†
Rabbit anti-NZB-MuLV antiserum	1000	1000
Heat stability		
10 min, 100°	1000	1000
30 min, 100°	1000	≥400 < 1000
pH stability		
pH 2, 20 hr, 4°	400	400
pH 12, 20 hr, 4°	400	<10
Perchloric acid	400	≥270
Ether extraction		
Aqueous phase	400	400
Residue of ether phase	400	<10
Ethanol/ether (3:1) extraction		
Residue of organic phase	1000	100
Dispersed precipitate	1000	<10
Chloroform/methanol (2:1) extraction		
Residue of chloroform phase	1000	<50

* Control serum samples were not heated or treated with acid, base, solvents or rabbit antiserum. Procedures were conducted as described in *Materials and Methods*.

† The titers given include the dilution resulting from the treatments employed.

inactivate the virus. The titer of NF was reduced 10-fold by extraction with ethanol/ether. The remaining activity was found in the resuspended residue of the organic phase (Table 1). After chloroform/methanol treatment, NF was inactivated.

Characterization of NF by enzymatic digestion

Proteases and Nucleases. Pronase, protease K, trypsin, and pepsin had no effect on NF in whole mouse sera (Table 2), nor did extraction of sera with ether after protease treatment. The NF in ether-extracted serum was also resistant to subsequent protease digestion. It was resistant to DNase and RNase before and after heating the sera to 100° (Table 2).

Lipid and Carbohydrate Hydrolases. α-Amylase treatment did not result in any decrease in NF, even when a concentration as high as 500 μg/ml was used (Table 2). Amylase treatment followed by Pronase also had no effect. Hyaluronidase, neuraminidase, and phospholipase A also did not reduce the level of NF in mouse sera (Table 2).

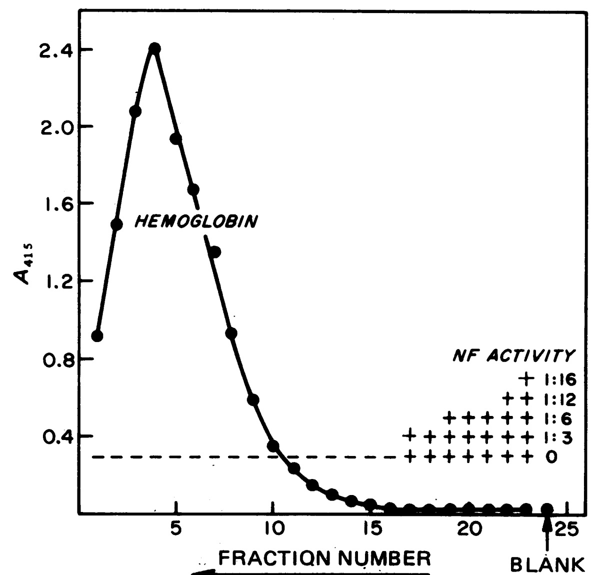

FIG. 1. Density gradient ultracentrifugation was performed with sucrose dissolved in 0.1 M NaCl, 1 mM EDTA, and 20 mM Tris-HCl at pH 7.4 (16). Serum samples, dialyzed against 0.01 M Tris at pH 7.4, were layered on preformed linear gradients of 5–20% sucrose. The samples were centrifuged in a Spinco SW 40 rotor, (39,000 rpm, 4°) in a Beckman L2 65B ultracentrifuge. Using hemoglobin as a marker in one tube, 24 fractions per gradient were collected and assayed for NF and protein content (18). The absorbance of each tube was measured at 415 nm to locate the hemoglobin. Each fraction was assayed for neutralizing activity.

Isolation of NF by sucrose gradient centrifugation and polyacrylamide gel electrophoresis

Initial attempts to determine the molecular weight of NF by sedimentation in sucrose density gradients showed that it remained at the top of the gradient (8). Prolonged centrifugation did not result in the migration of NF into the gradients. A gradient profile, representative of eight experiments, is shown in Fig. 1. Hemoglobin (sedimentation coefficient 5.5 S) is near

Table 2. Sensitivity of neutralizing factor to enzymatic digestion

Treatment	Neutralizing titer			Sensitivity
	Control sera*	Control sample†	Experimental sample‡	
Pronase, 100 μg/ml	1000	40	1000	Resistant
Protease K, 250 μg/ml	1000	<10	1000	Resistant
Pepsin, 100 μg/ml	1000	<10	1000	Resistant
Trypsin, 10 μg/ml	400	<10	400	Resistant
DNase, 100 μg/ml	100	0	100	Resistant
RNase, 100 μg/ml	100	0	100	Resistant
RNase, after heating to 100°	100	0	100	Resistant
Amylase, 500 μg/ml	1000	0	800	Resistant
Hyaluronidase, 3 units	1000	0	1000	Resistant
Neuraminidase, 50 units	1000	0	1000	Resistant
Phospholipase A, 500 μg/ml	1000	0	1000	Resistant

* The control sera were obtained from NZB, (CBA × C57)F_1, CBA, and C3H mice. The NF titer given is for untreated sera.

† Control sample assessed the effect of the enzyme and diluent alone on the virus titer.

‡ Experimental sample gives the NF titer of enzyme-treated sera. All samples were digested with the given concentration of enzyme for 1–2 hr in a 37° water-bath.

Table 3. Separation of antixenotropic virus neutralizing factor by sucrose density gradient centrifugation

Sample	Volume (ml)	Neutralizing titer	Protein concentration (mg/ml)	Total protein (mg)	Total activity	Specific activity (units/mg of protein)	Recovery (%)
Pooled NZB sera	1.34	1:400	67	90	536	6.0	100
First sucrose gradient pool	10.93	1:20	0.115	1.26	218.6	173.5	40.8
Second sucrose gradient pool	7	1:20	<0.01	<0.07	⩾140.0	⩾2000	⩾26.2

Procedure is explained in *text*.

the bottom of the tube and NF is confined to the top region. A partial purification of NF was obtained by two successive centrifugations of serum in 5–20% gradients. The specific activity was increased from 6 units/mg to more than 2000 units/mg and approximately one-fourth of NF was recovered in this isolation procedure (Table 3). NF in this partially purified preparation was unstable to freezing and thawing and its activity was markedly reduced after 30 days at −70°.

We determined that NF was not affected by polyacrylamide or the concentration of sodium dodecyl sulfate present in the fraction that contained neutralizing activity. Activity was found at the anodal end of the gel near the bromophenol blue dye front. A light protein band was detected by Coomassie blue staining in that region. Periodic acid/Schiff staining failed to demonstrate the presence of glycoprotein. After purification from the gel the neutralizing activity remained specific for xenotropic virus and had no effect on XC plaque formation by ecotropic AKR-MuLV. Fractions from a control gel containing no sera did not neutralize MSV(NZB-MuLV).

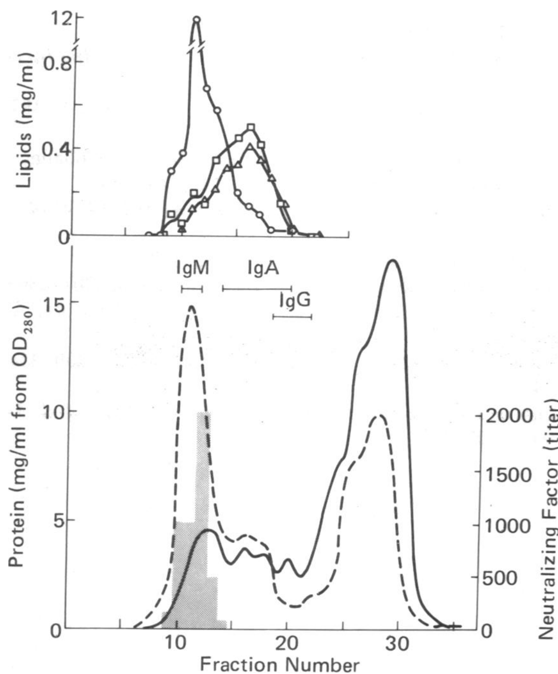

FIG. 2. Gel permeation chromatography of whole mouse serum on Sephadex G-200. For analyses of the fractions, see *Materials and Methods*. Lower portion of figure, (—) protein, (- - -) OD_{280}, stippled bars indicate neutralizing titer; brackets indicate fractions in which IgM, IgA, and IgG were present. Upper portion, lipid content of fractions: (O) triglyceride, (□) cholesterol, (△) phospholipid.

Gel permeation chromatography

The pattern of elution of proteins of mouse serum from Sephadex G-200 is shown in Fig. 2. The peak of optical density at 280 nm in fractions 8–13 is primarily attributable to light scattering by the large triglyceride-bearing lipoproteins [chylomicrons and very low density lipoproteins (VLDL)] which were observed in agarose gel electrophoretograms of those fractions. Lipoproteins of beta and alpha mobility appeared in fractions 14–19, where the bulk of the cholesterol and phospholipid were found, corresponding to the low density (LDL) and high density (HDL) lipoproteins, respectively. NF was eluted with the lipoproteins and IgM, well separated from IgA, IgG, and most other serum proteins.

Ultracentrifugal distribution of NF in mouse serum

The supernatant ($d < 1.21$ g/cm^3) and infranatant ($d > 1.21$ g/cm^3) fractions of an NZB serum were tested for NF. As illustrated in Table 4, neutralizing activity was found only in the fraction containing the lipoproteins ($d < 1.21$ g/cm^3). The quantity of NF recovered approached that present in unfractionated serum. This supernatant fraction was shown by agarose gel electrophoresis (21) to contain lipoproteins of alpha, prebeta, and beta mobility. When stained for protein with amidoschwartz 10B (E. Merck), bands were only seen in the regions corresponding to these lipoproteins. Mouse albumin and immunoglobulins were undetectable in this fraction by immunodiffusion.

DISCUSSION

We have described a soluble nonimmunoglobulin factor in normal mouse serum that specifically neutralizes endogenous murine xenotropic virus (8). This neutralizing factor (NF) is detected at various concentrations in mouse sera and is not at a constant level in any one strain of mice. It varies in titer from

Table 4. Association of neutralizing factor with the lipoprotein fraction of mouse serum

Sample	Density (g/cm³)	Neutralizing titer: Anti-xenotropic	Neutralizing titer: Anti-ecotropic
NZB serum	Unfractionated	1000	<10
Serum			
Fraction 1	<1.21	800	<10
Fraction 2	>1.21	<10	<10

Serum was fractionated by preparative ultracentrifugation as described in *text*. Neutralization tests were conducted as described in *Materials and Methods*.

1:10 to 1:10,000. Sera from NZB mice have contained the highest titer (1:10,000), but other mouse strains may have titers as high as 1:4,000 (4, 8).

We have demonstrated that NF is not an immunoglobulin (8). Ultracentrifugal separation of immunoglobulins and lipoproteins in the present study confirms this observation, and demonstrates the association of NF with the serum lipoproteins. By gel permeation chromatography it is found in the region of lipoproteins and IgM, which explains the previous conclusion by others that it was an immunoglobulin (23). The factor is not the 70,000 molecular weight glycoprotein(s) that circulates in high titer in mouse serum (24, 25). This protein would be pelleted after 113 hr in a sucrose gradient (Fig. 1), would stain with periodic acid/Schiff reagent in gels, and would be neutralized by anti-NZB-MuLV antiserum (Table 1). Moreover, it would probably be sensitive to some of the enzyme digestions we employed. Preliminary studies employing ultracentrifugal fractionation of mouse lipoproteins place the bulk of antixenotropic virus neutralizing activity in the density interval 1.019–1.175 g/cm^3. This range transcends the discriminating density for mouse high density and low density lipoproteins (1.063 g/cm^3). Moreover, the apparent particle diameter of NF exceeds that of high density lipoproteins and most low density lipoproteins. Further studies are required to determine whether NF is associated with one of the major classes of lipoproteins or a specific minor lipoprotein species within this density interval.

The sensitivity of NF in whole serum to solvent extraction parallels that of the major serum lipoproteins, which are largely resistant to extraction with relatively apolar solvents such as ether alone (26, 27). The partial recovery of NF after ethanol/ether extraction suggests that protein may be important for neutralization, since this solvent system, unlike chloroform/methanol, solubilizes small amounts of apolipoproteins (28). Further, like NF, lipoproteins are somewhat resistant to attack by proteases, presumably due to lipid–protein interaction (29). However, resistance of NF in whole serum to enzymatic treatment may be due to the competitive effect of other substrates. When NF is purified further, studies employing enzymic digestion may yield very useful information.

A regulatory effect on cell-mediated immunity has been reported for low density lipoproteins in human serum (30). These lipoproteins block T (thymus-derived) cell activation and mixed lymphocyte reactions. We have now recognized a factor associated with mouse serum lipoproteins that specifically inactivates a virus and represents a nonimmunoglobulin neutralizing reaction of a host to an endogenous virus. NF may also have a regulatory role. By interacting with viral antigens on cell surfaces, it may affect normal cellular functions (10, 11).

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