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Apolipoprotein is responsible for neutralization of xenotropic type C virus by mouse serum

(virus infectivity/lipoproteins)

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ABSTRACT **We have shown that the circulating lipoproteins of the mouse contain a potent inhibitor of infectivity of the xenotropic type C virus. This virus neutralization does not involve immunoglobulins or complement. After fractionation of the lipoproteins on the basis of particle size, flotation properties, and electrostatic charge, virus neutralizing activity is found primarily in the triglyceride-rich lipoproteins (predominantly the chylomicrons) and in the HDL_2 subfraction of the high density lipoproteins. In fasted animals, activity resides chiefly in the high density lipoproteins. Neutralization titers increase strikingly during alimentary lipemia in both the lipoproteins of the $\rho < 1.006$ g/cm^3 fraction and the high density lipoproteins. Increased activity persists in the high density lipoproteins after the lipemia recedes. Virus neutralizing activity is completely eliminated in all fractions by antiserum against high density lipoproteins and, in the triglyceride-rich fractions, by antiserum to murine apolipoprotein B. Complete removal of lipids markedly reduces the neutralizing activity of both classes of lipoproteins. Apolipoproteins delipidated with tetramethylurea retain some activity, which is enhanced by binding to a phospholipid-stabilized triglyceride emulsion and which is abolished by proteolytic digestion. We have demonstrated *in vitro* transfer of activity between high density and very low density lipoproteins of the mouse. These data indicate that xenotropic virus neutralization by normal mouse serum depends upon a protein that transfers among lipoprotein particles in a fashion analogous to the C apolipoproteins of other mammalian species.**

DNA copies of the genomes of mouse type C RNA viruses are integrated into mouse chromosomes and are passed to successive generations (1). In the mouse, two major classes of these endogenous type C murine leukemia viruses have been defined by their host range. The ecotropic virus readily infects mouse cells and produces malignancies in mice (1–3), whereas the xenotropic virus productively infects only cells from heterologous mammalian and avian species (2–5).

We have observed potent inhibition of infectivity of xenotropic virus, but not ecotropic virus, by mouse serum, and have shown that this activity does not involve immunoglobulins (6, 7), a finding confirmed by others (8). Furthermore, we have found that neutralizing activity (NA) against the xenotropic virus resides completely with the serum lipoproteins (7). We have chosen the general term virus neutralization because the fate of the virus after interaction with lipoprotein is unclear.

In the present study we have demonstrated that this NA is contained in several classes of circulating lipoproteins, that it requires an intact protein component, and that it can be transferred among lipoproteins in a manner similar to the transfer of certain apolipoproteins.

MATERIALS AND METHODS

Neutralization Assays. Neutralization assays were conducted as described (6, 7). NA against xenotropic virus was measured by reduction of focus formation in cultured normal rat kidney (NRK) cells by a pseudotype of murine sarcoma virus that contains in its envelope the xenotropic virus type-specific antigen (6, 7). NA against ecotropic virus was measured by a reduction of X-C plaque formation (9) in NIH Swiss mouse embryo cells by the AKR-L1 murine leukemia virus (obtained originally from J. W. Hartley, Bethesda, MD and propagated in NIH Swiss mouse embryo cells). In both assays, cells were also inoculated with virus that had been incubated with cell culture medium alone as a control. The titer of neutralization was defined as the reciprocal of the highest dilution that reduced the number of foci or plaques observed by 67%, compared to the virus controls.

Animals. Serum was prepared fresh from blood drawn from NFS (from the National Institutes of Health) and (C57 Bl/6 × C3H)F_1 mice [$(B_6C_3)F_1$] (Simonsen Laboratories, Gilroy, CA) that had been fed mouse chow (Ralston Purina, St. Louis, MO), mouse chow soaked in olive oil, or a formula diet enriched in fat [37.5% lard/12.5% butter/30% casein/vitamins/minerals/cellulose (Univ. of California, Animal Care Facility)]. Some sera from $(B_6C_3)F_1$ mice were given to us by J. Ihle, Frederick, MD. Results with frozen sera did not differ from those obtained with fresh sera.

Ultracentrifugation. Lipoproteins were separated from serum by preparative ultracentrifugation according to a modification of the method of Havel *et al.* (10). Nonprotein solvent density was adjusted by using ^2H_2O and KBr to permit the sequential separation of lipoproteins at the discriminating densities: $\rho \leq 1.006$, 1.063, and 1.21 g/cm^3. Each ultracentrifugation was performed at 12°C at 105,000 × *g* for 24 hr in a Beckman model L3-40 ultracentrifuge. For efficient separation of the lipoprotein subclasses, ultracentrifugation was repeated once at $\rho = 1.063$ and at $\rho = 1.21$ g/cm^3. Gentamycin sulfate (10 μg/ml), sodium azide (0.5 μg/ml) and EDTA (0.4 μg/ml) (pH 8.6) were present in the ultracentrifugal media. The $\rho < 1.006$ g/cm^3 fraction contained chylomicrons and lipoproteins of pre-β electrophoretic mobility [very low density lipoproteins (VLDL)]. The $1.006 < \rho < 1.063$ g/cm^3 fraction [low density lipoproteins (LDL)] contained only lipoproteins of β mobility and the $1.063 < \rho < 1.21$ g/cm^3 fractions [high density lipoproteins (HDL)] showed only α mobility when examined by electrophoresis in agarose gels (11). Ultracentrifugal fractions were dialyzed against 0.15 M NaCl before

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Abbreviations: VLDL, LDL, and HDL, very low density, low density, and high density lipoproteins, respectively; TMU, 1,1,3,3-tetramethylurea; apo-B, apolipoprotein B; NA, neutralizing activity; $(B_6C_3)F_1$, (C57 Bl/6 × C3H)F_1 mice.

studying their interaction with virus. The lipoprotein fractions remained active after 1 month of storage at −70°C.

Gel Permeation Chromatography. Whole serum and dialyzed ultracentrifugal fractions were subjected to gel permeation chromatography at 1°C on 0.6 × 42.5-cm columns of 4% crosslinked agarose (Pharmacia). Lipoproteins were eluted with 0.15 M NaCl/gentamycin (10 μg/ml)/EDTA (0.1 mM) for assay of neutralizing activity. This concentration of EDTA, employed to inhibit hydroperoxidation of lipids, had no effect on the virus or the cultured cells.

Polyanion Precipitation. Lipoprotein fractions were selectively removed from mouse serum by precipitation with heparin and divalent cations (12). Two methods of precipitation were used: 0.01% heparin (from porcine intestine, Riker Laboratories, Northridge, CA) plus 0.05 M $MnCl_2$ and 0.25% heparin plus 0.1 M $MgCl_2$.

Separation of Lipoprotein Constituents. The virus NA of isolated serum lipids was examined by extraction of whole serum and of lipoprotein fractions with organic solvents. Extractions were performed for 16 hr with 20 vol of either diethyl ether at 4°C; ethanol/diethyl ether, 3:1 (vol/vol), at 20°C; or chloroform/methanol, 2:1 (vol/vol), at 20°C. An equal volume of water was added to the latter and the phases were allowed to separate. The organic phases of the extractions were evaporated to dryness under N_2 and the residues were dispersed by sonication in 0.15 M NaCl/0.1 mM EDTA for assay of NA. The aqueous phases from the ether and chloroform/methanol extractions were also tested. The effect on infectivity of both xenotropic and ecotropic viruses of a number of naturally occurring lipids in various dispersed states was also studied. These preparations included a phospholipid-stabilized triglyceride emulsion (Intralipid, Vitrum, Stockholm); phosphatidylcholine vesicles (5 μg/ml) prepared with a French pressure cell (R. L. Hamilton, personal communication); and sonicated dispersions of phosphatidylglycerol (10 μg/ml), bovine heart cardiolipin (1 μg/ml), soybean phosphatidylinositol (2 μg/ml), dioleylphosphatidylethanolamine, DL-cephalin, and DL-phosphatidic acid (all 1 μg/ml), sphingomyelin from bovine serum (2 μg/ml), and gangliosides (type III) and cerebrosides (1 μg/ml), both from bovine brain.

NA of the protein moieties of serum lipoproteins was also evaluated. The protein moiety of HDL was delipidated by the techniques of Lux *et al.* (13) and Scanu and Edelstein (14), and both HDL and triglyceride-rich lipoproteins were extracted with 1,1,3,3-tetramethylurea (TMU) (15, 16). TMU was removed by dialysis in 0.15 M NaCl. The precipitated proteins of HDL (13, 14) were dissolved in 0.15 M NaCl at 0.5 mg/ml. The TMU-extracted apoproteins were also dissolved in 0.15 M NaCl. The resolubilized apolipoproteins were tested for NA before and after incubation for 30 min at 42°C with Intralipid (100 mg/ml) or with phosphatidylcholine vesicles (3 mg/ml).

To determine whether latent NA might be detected in the lipoprotein-free ultracentrifugal infranatant fraction ($\rho > 1.21$ g/cm^3), this fraction was incubated after dialysis with Intralipid at 42°C for 30 min. The lipid phase, recovered by ultracentrifugation at 105,000 × *g* for 2 hr, was tested for NA.

Transfer of NA. We studied the transfer of NA from lipoproteins of the $(B_6C_3)F_1$ mouse to those of the NFS mouse, which normally has little or no virus NA. $(B_6C_3)F_1$ VLDL were incubated at 37°C for 16 hr with inactive HDL from the NFS mouse at concentrations equal to those in the original sera. Similar experiments were conducted with inactive NFS VLDL and active $(B_6C_3)F_1$ HDL. Moreover, $(B_6C_3)F_1$ HDL was incubated with Intralipid. After incubation, the fractions were separated ultracentrifugally at a density of 1.006 g/cm^3, dialyzed against 0.15 M NaCl/0.1 mM EDTA, and tested for NA. The apo-HDL and apo-VLDL solutions (both 1 mg/ml) were incubated with 0.1 vol of Intralipid for 30 min at 44°C. The Intralipid phase was recovered ultracentrifugally, as described above.

Proteolytic Attack. Apolipoproteins prepared by extraction of the $\rho < 1.006$ g/cm^3 fraction of serum from fat-fed $(B_6C_3)F_1$ mice with TMU (protein content 2.5 mg/ml) specifically neutralized at a dilution of 1:10 the infectivity of xenotropic but not ecotropic virus. A portion of this apoprotein preparation was incubated with 0.04 mg of type VI protease from *Streptomyces griseus* (Sigma) per ml for 2 hr at 37°C. A control portion was incubated with buffer alone. Dilutions of 1:10 or greater of buffer containing the enzyme did not inhibit viral infectivity; hence, the treated lipoproteins were tested at those dilutions. Before being assayed for NA, the apoproteins were complexed with 0.1 vol of Intralipid at 42°C for 30 min.

Immunologic Studies. Antibodies were raised in New Zealand White rabbits to whole HDL and VLDL-chylomicron fractions from $(B_6C_3)F_1$ mice and to apolipoprotein B (apo-B) obtained by extraction of $\rho < 1.063$ g/cm^3 lipoproteins of the NFS mouse with 4.2 M TMU, followed by two extractions with 3:1 ethanol/diethyl ether.

RESULTS

Distribution of NA Among Lipoproteins Separated by Ultracentrifugation. Ultracentrifugal fractionation of 10 serum specimens obtained at random from $(B_6C_3)F_1$ and NZB mice always showed substantial activity among the HDL ($1.063 < \rho < 1.21$ g/cm^3). There was usually little or no activity among the LDL ($1.006 < \rho < 1.063$ g/cm^3). However, the activity of the $\rho < 1.006$ g/cm^3 lipoproteins (VLDL and chylomicrons) showed wide variation. Some sera had little or no activity whereas others had levels as high as 1:1000. The acute effects of fat-enriched diets were therefore evaluated to determine whether this variation might be due to alimentary lipemia. The end-titer NA of five whole serum pools from groups of six animals on regular chow was generally about 100. After 1 day on a fat-enriched diet, the NA of the whole serum pools increased by approximately 10-fold, and in one instance 40-fold, to a titer of 1:4000. The distribution of activity among lipoprotein fractions obtained by ultracentrifugation of a representative pair of pools obtained from one group of six mice is shown in Table 1. On a control diet the activity in the $\rho < 1.006$ g/cm^3 fraction was very low, and the bulk of the activity resided in the HDL. With fat-feeding, however, the activity of the $\rho < 1.006$ g/cm^3 fraction increased 40-fold, and that of the HDL, 5-fold. In both pools the activity of the LDL fraction was minimal and no activity was found in the $\rho > 1.21$ g/cm^3 fraction, which is virtually devoid of lipoproteins. This is consistent with our observations on the $\rho > 1.21$ g/cm^3 fraction from 20 different serum pools, in which NA was totally absent in 16 and was found only in undiluted infranate in the re-

Table 1. Effect of fat feeding on distribution of xenotropic virus NA in mouse serum

Diet	Whole serum	End titer NA in lipoprotein fraction (g/cm^3)			
		<1.006	1.006–1.063	1.063–1.21	>1.21
Control*	100	10	10	40	0
Chow with olive oil supplement	1000	400	1	200	0

* Pools of serum from six $(B_6C_3)F_1$ mice.

maining 4 samples after only a single ultracentrifugation. The distribution of NA within the HDL interval was further studied by subfractionation at a density that separates the HDL_2 and HDL_3 subspecies in human serum. In two pools from chow-fed and one from fat-fed animals, over 95% of the activity of HDL was found in the HDL_2 subfraction ($1.063 < \rho < 1.175$). Mouse immunoglobulins were undetectable by immunodiffusion in any of the lipoprotein fractions.

Gel Permeation Chromatography of NA. To determine whether the hydrodynamic volumes of the particles with which NA is associated correspond to those of serum lipoproteins, whole serum and ultracentrifugal fractions were studied by gel permeation chromatography. The results of chromatography on a 4% agarose gel of pooled whole serum samples from mice fasted for 12 hr after being fed regular chow, nonfasting mice fed a fat-rich diet, and mice fasted 12 hr after eating the fat-rich diet are shown in Fig. 1. These data are representative of the results of three such experiments. NA in the mice fasted after a regular chow meal was limited to particles of the diameters of HDL and LDL. The optical scan of the eluate indicated that there was little triglyceride-rich lipoprotein present. The serum from fat-fed animals contained a visible chylomicron layer when refrigerated overnight. This serum showed a large tailing peak at the void volume, which contained NA up to 1:1000 dilution (corresponding to 1:3000 in serum) and showed a moderate increase in activity in the region containing the larger HDL and the LDL. A fast of only 12 hr resulted in disappearance of activity in the region about the void volume. However, the activity in the region of HDL was 10-fold higher than in serum from animals fed regular chow.

When a $\rho < 1.006$ g/cm^3 fraction containing substantial NA was chromatographed on this column, the activity appeared only in the void volume peak. When an active HDL was applied to the column, activity was eluted only in the region around 25 ml (≈85 Å). Both the void volume and HDL peaks were free of mouse albumin by immunodiffusion. Neither mouse IgG, IgA, nor IgM could be detected in the void volume region; however, immunoglobulins were found in the HDL region when whole serum was applied to the column, as expected.

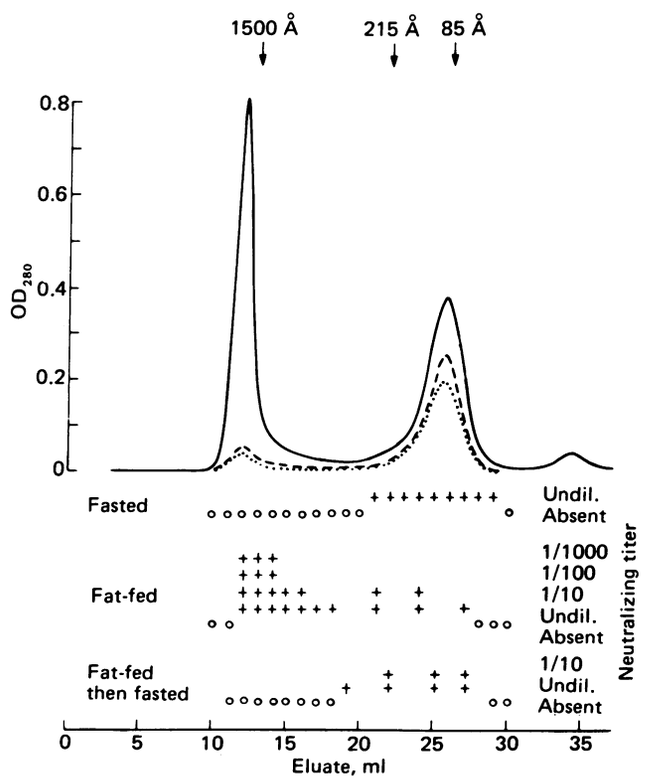

FIG. 1. Gel permeation chromatography of 0.2 ml of pooled whole serum from $(B_6C_3)F_1$ mice on a 0.6 × 42.5-cm column of Sepharose CL-4B. Flow rate, 1.25 ml/hr. ·····, Animals on control diet and fasted 12 hr before the experiment; —, fat-fed animals, - - -, animals fed 50% fat diet for 4 days and then fasted for 12 hr. Particle diameters indicated with arrows denote elution volumes of purified human lipoproteins of known particle diameters used as calibrants.

Mobility of NA upon Electrophoresis in an Agarose Gel. To evaluate the charge of the particles with which NA is associated, we eluted lipoproteins from agarose gel electrophoresis strips. When fasting serum was applied, NA was found in the pre-β and α regions, consistent with the mobility of VLDL and HDL, respectively. It was eluted from the same regions when the $\rho < 1.006$ g/cm^3 and HDL fractions were subjected to electrophoresis after dialysis. When nonfasting serum was applied, NA was found in the α region and also in a broad zone from the origin through the pre-β region. The latter is consistent with the mobility of chylomicrons.

Changes in NA Associated with Polyanion Precipitation of Lipoproteins. Heparin/Mn^{2+} treatment precipitates all lipoproteins containing apo-B (chylomicron, VLDL, and LDL) (12) and a minor subfraction of HDL which contains the arginine-rich apoprotein. After exposure to 0.01% heparin and 0.05 M Mn^{2+}, the titer of NA in a pool of serum from nonfasting $(B_6C_3)F_1$ mice fell from 1:3000 to 1:100. Because this titer is lower than that observed in the HDL fraction prepared ultracentrifugally from the same pool (1:300), it appears that an active subfraction of the HDL was precipitated along with the lipoproteins containing apo-B. Precipitation with heparin (0.25%) and 0.1 M Mg^{2+} completely spared the NA found in the HDL ultracentrifugal interval. These findings are consistent with precipitation by heparin/Mn^{2+} of a subfraction of HDL, probably bearing the arginine-rich apoprotein, with which the majority of the activity of HDL is associated.

Effects of Solvent Extraction and of Lipid Emulsions. Treatment of a pool of serum (titer 1:2000) from $(B_6C_3)F_1$ mice with ethanol in the cold had no effect on its activity against xenotropic virus, whereas neither the lipid nor protein moieties suspended in saline, after extraction of the serum by chloroform/methanol, had any detectable virus inhibitory effect. Moderate neutralization of xenotropic but not ecotropic virus (1:200 end titer) was observed with the resuspended solids recovered from ethanol/ether extraction. The suspension of the residue insoluble in that solvent had no effect on either virus.

Intralipid and the sonicated dispersions of phosphatidylcholine, phosphatidic acid, cephalin, dioleylphosphatidylethanolamine, cerebrosides, gangliosides, and sphingomyelin had no effect on either virus. Slight inhibition (only in the initial concentration) of xenotropic virus was noted with phosphatidylglycerol and phosphatidylinositol. The suspension of cardiolipin inhibited the infectivity of xenotropic virus at 0.05 mg/dl and ecotropic virus at 0.5 mg/dl.

Combination of Apolipoproteins with Ordered Lipid: Transfer of NA to Intralipid. Apo-HDL prepared from active $(B_6C_3)F_1$ HDL by the method of either Lux *et al.* (13) or Scanu and Edelstein (14) had no activity against either virus alone or after incubation of the apoproteins with phosphatidylcholine vesicles. Some activity (end titer 1) against xenotropic virus was detectable in apoproteins extracted with TMU from the chylomicron-VLDL and HDL fractions of serum. This neutralizing activity of both apoprotein fractions was enhanced 10-fold when 0.1 vol of Intralipid was added to the solution.

Transfer of NA among Lipoproteins. After incubation of

Table 2. Transfer of NA against xenotropic virus to Intralipid

Incubation mixture	Neutralizing activity (end titer)* after reisolation of Intralipid	
	$\rho < 1.006$ g/cm^3	$\rho > 1.006$ g/cm^3
HDL alone	<1	100
HDL plus Intralipid	40	10
Intralipid alone	<1	—
$\rho > 1.21$ g/cm^3 infranate of serum†	—	<1
$\rho > 1.21$ g/cm^3 infranate† plus Intralipid	1	<1
$\rho > 1.21$ g/cm^3 infranate of serum‡	—	<1
$\rho > 1.21$ g/cm^3 infranate‡ plus Intralipid	1	<1

* All neutralization titers were determined in duplicate.

† Prepared from pooled serum of six $(B_6C_3)F_1$ mice that were fat-fed.

‡ Prepared from pooled serum of six $(B_6C_3)F_1$ mice that were fasted for 12 hr.

active $(B_6C_3)F_1$ HDL (end titer, 10) with VLDL from NFS mice (end titer, <1) the end titers of the VLDL and HDL were 10 and 1, respectively, indicating that substantial transfer of NA had occurred. Furthermore, incubation of $(B_6C_3)F_1$ VLDL (end titer, 100) with NFS HDL (end titer, <1), in concentrations found in mouse serum, led to transfer of NA to the NFS HDL.

Incubation of $(B_6C_3)F_1$ HDL with Intralipid resulted in the transfer of substantial amounts of NA to the phospholipid/triglyceride emulsion (Table 2). Application of this technique to the lipoprotein-free ultracentrifugal infranate fraction of serum ($\rho > 1.21$ g/cm^3) suggested that some latent antixenotropic activity was present that could only be demonstrated by association with lipid.

Proteolytic Attack on NA. Pronase digestion of TMU-soluble apoproteins from the $(B_6C_3)F_1$ VLDL-chylomicron fraction abolished the NA that otherwise was observed at an end titer of 10 in control cultures inoculated with the Intralipid complex apolipoprotein.

Inhibition of NA by Antilipoprotein Antibodies. A 1:5 dilution of rabbit antiserum raised against $(B_6C_3)F_1$ HDL completely inhibited the NA of active $(B_6C_3)F_1$ serum (end titer, 1:1000) at a 1:10 dilution. This rabbit antiserum inhibited the NA of both purified HDL and VLDL-chylomicron fractions at similar titer. Antiserum to mouse apo-B inhibited the NA of LDL and VLDL-chylomicron fractions from $(B_6C_3)F_1$ mice but had no effect on the activity of HDL. The anti-HDL serum showed a major precipitin line with mouse HDL and a faint line of partial identity when diffused against mouse VLDL, whereas the anti-apo-B serum reacted only with those lipoproteins known to contain apo-B—i.e., LDL, VLDL, and chylomicrons.

DISCUSSION

The consistent demonstration of NA specifically against the xenotropic type C virus in ultracentrifugal fractions that contain only mouse serum lipoproteins confirms the lipoprotein character of the neutralizing factor. The absence of detectable mouse immunoglobulins in these fractions indicates that the activity is not due to binding and flotation of immunoglobulins by lipoproteins (6, 7). The data presented in this report indicate that NA is associated with particles that have the same flotation properties, particle diameters, and electrophoretic charges as do serum lipoproteins and that activity is associated with more than one class of serum lipoproteins, predominantly the triglyceride-rich particles of $\rho < 1.006$ g/cm^3 and the HDL_2 subfraction ($1.063 < \rho < 1.175$ g/cm^3).

Selective inhibition of NA in LDL and in $\rho < 1.006$ g/cm^3 fractions by antiserum to apo-B indicates that NA in fact is an integral part of the apo-B-containing lipoproteins. Inhibition of NA in both HDL and triglyceride-rich lipoproteins by antiserum to active HDL is consistent with the presence of common (non-apo-B) determinants in these lipoproteins (17–19). Whether this latter antiserum contains immunoglobulin directed specifically at the neutralizing factor is not yet determined because it is likely that, if an antiserum reacts with any determinant in a lipoprotein complex, it would inhibit the activity of the neutralizing factor. Abolition of NA by proteolytic digestion demonstrates that a protein is required for the virus neutralization. The presence of NA in extracts obtained with TMU, which excludes apo-B, and the presence of NA in HDL, which contains no apo-B, indicates that this apoprotein is not responsible for neutralization. Dispersed lipids are relatively ineffective alone. Virus neutralization is enhanced when the protein factor is associated with a phospholipid/triglyceride emulsion and maximal when the association between protein and lipid constituents of the native lipoproteins is undisturbed.

The increase in the titer of NA after fat feeding suggests that the wide variations in NA in mouse sera (6) may be diet-related. The appearance of large amounts of NA among the triglyceride-rich lipoproteins after fat feeding could result from its secretion with nascent chylomicrons. However, the possibility of activation of latent activity within the plasma compartment during alimentary lipemia cannot be evaluated until a quantitative assay for the protein is available. During alimentary lipemia, activity in HDL also increases. With fasting, it persists beyond the disappearance of activity among the triglyceride-rich lipoproteins. Such behavior is compatible with a transfer of apoproteins to HDL similar to the migration of C apolipoproteins during the catabolism of chylomicrons and VLDL in man and the rat (17–19). The transfer of NA between HDL and triglyceride-rich lipoproteins *in vitro* is consistent with such migratory behavior. It is probable that the distribution of the factor is determined both by its absolute affinities for the triglyceride-rich lipoproteins and for HDL and by the relative concentrations of the lipoproteins. Just as the affinity of certain C apolipoproteins for VLDL remnants declines as triglyceride hydrolysis proceeds (20), it is likely that the transfer of the neutralizing factor to HDL depends on catabolism of chylomicrons and VLDL.

This neutralization of an endogenous type C virus shows that specific antiviral activity need not involve immunoglobulins. A suppressor role for human lipoproteins in the control of the cellular immune response has recently been suggested (21, 22). This activity in human sera appears to be different from the mouse virus neutralizing factor, particularly because the former is most abundant among the LDL, whereas the latter is almost entirely excluded from those lipoproteins. Because of the ubiquity of endogenous type C viruses and their association with normal and malignant tissues, interaction with circulating lipoproteins may represent an important regulatory mechanism in the host (23).

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