

Dietary Hepatic Cholesterol Elevation: Effects on Coxsackievirus B Infection and Inflammation

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Mice made hypercholesterolemic (HC) by diet are highly susceptible to coxsackievirus (CV) B5, whereas normal adult animals remain resistant. In attempting to define those dietary-induced physiological changes which contribute to altered resistance, a strong association between accumulation of intrahepatic cholesterol and increased CV B5-induced mortality was demonstrated, with maximum susceptibility to CV coinciding with a 2.5-fold increase in the ratio of hepatic cholesterol to protein. This metabolic imbalance was associated with a lower clearance rate of CV from the blood and liver of C57BL/6 mice, although virus-specific neutralizing antibody production was unaltered. In addition to CV, HC mice were more susceptible to an intravenous inoculation of *Listeria monocytogenes* in comparison to controls. The macrophage stimulant *Corynebacterium parvum* failed to increase resistance of HC mice to a high dose of CV B4 and *L. monocytogenes* and failed to induce the hepatomegaly, splenomegaly, and cellular infiltrate seen in the liver and spleen of normal animals. Furthermore, the peritoneal monocytic infiltrate induced by thioglycolate in normal animals was absent in HC mice. Results from these experiments suggest that decreased resistance to CV in the HC host is attributed to a defect in the nonspecific immune responses of macrophages and monocytes which are of primary importance in resistance to this virus and other infectious agents.

The potentiating effect of a high cholesterol diet on the susceptibility to and severity of a coxsackievirus (CV) B5 infection in the adult mouse has been well documented (4, 15, 16). Infection of hypercholesterolemic (HC) mice results in mortality and high titers of CV B5, which persists in target organs, causing severe pathology often unaccompanied by inflammatory cells. However, the mechanism of this altered resistance to CV has not been elucidated from the complex metabolic and possible immunological changes accompanying the HC state.

The macrophage and mononuclear inflammatory cells are of primary importance in resistance to CV in the mouse (18, 23). Although virus-specific antibody administered in high doses protects susceptible animals from a CV infection, diluted preparations of immune sera require concurrent transfer of macrophages for effective protection (18). Furthermore, resistance to CV can be reduced by inhibitors of mononuclear cell functions without changes in virus-neutralizing antibody formation (23), suggesting that macrophages and monocytes act alone or in conjunction with antibody to suppress CV infections. Evidence to date suggests that thymic lymphocytes are not involved in initial resistance to this virus (24).

The objective of this research was first to identify a specific physiological alteration induced by dietary cholesterol which correlates with decreased viral resistance and second to define the immunological defect(s) associated with such a metabolic change. When various physiological parameters and virus-induced mortality were concomitantly measured at weekly intervals during consumption of a high-lipid (HL) diet, the mortality rate was associated with an elevated hepatic cholesterol/protein ratio. Subsequently, liver macrophages from HC mice were found to be deficient in antiviral and bactericidal activities. This defect appears to involve more than a simple hepatic blockade, as the monocytic and polymorphonuclear leukocytic (PMN) chemotactic responses at an inflammatory site were also inhibited in HC mice. This report demonstrating the inhibitory effect of a HL diet on nonspecific macrophage and monocyte functions in vivo may have important implications in resistance to numerous infectious agents and to tumorigenesis.

MATERIALS AND METHODS

Animals. Male, inbred C57BL/6J were obtained at 6 weeks of age from Jackson Laboratory, Bar Harbor, Me. Upon arrival, all mice were fed Purina laboratory

chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum. At 7 weeks of age, experimental animals were fed an HL diet for 10 weeks, whereas control mice were continued on the same chow feed. After 10 weeks on the HL diet, all remaining experimental animals were returned to the laboratory chow diet. The HL diet was a modification of that previously reported (14–16) and contained 52% sucrose, 18.5% casein, 18% lard, 1% cholesterol, 0.5% cholic acid, and vitamin supplements.

Biochemical assays. Plasma-free cholesterol, cholesterol esters, fatty acids, and triglycerides were separated and quantitated (milligrams of lipid per deciliter of plasma) by a thin-layer chromatography procedure previously described (10, 15, 16). Hepatic lipids were extracted from approximately one gram of tissue using the method of Bligh and Dyer (3) and were quantitated (milligram of lipid per gram of liver) by the above procedure. Total protein from plasma and homogenized liver were quantitated by the method of Lowry et al. (17).

Plasma aspartate transaminase and alkaline phosphatase activities and bilirubin were assayed as an indication of liver function. The enzyme activities were quantitated (international units per liter) colorimetrically using corresponding Beckman Liquid-STAT reagent kits (Beckman Instruments, Inc., Fullerton, Calif.). Plasma total bilirubin was quantitated (milligrams of bilirubin per deciliter of plasma) spectrophotometrically, using the Hycel bilirubin test kit (Hycel, Inc., Houston, Tex.).

Cell culture. HeLa cells used for preparation of virus pool and for virus titrations were obtained from Flow Laboratories, Rockville, Md., and were propagated as previously described (4).

Virus. The Grenier strain of CV B5, originally isolated from a patient with aseptic meningitis and previously used in similar studies (1, 3), was plaque-purified in HeLa cells by the procedure of Cole et al. (6). The strain of CV B4 used in these studies has also been previously described (22). All virus quantitations were carried out by a methyl cellulose-overlay plaque technique of HeLa cell monolayers.

Virus susceptibility. Mice were inoculated intraperitoneally (i.p.) with 1×10^9 PFU of CV B5 after consuming the HL diet for 1, 2, 3, 4, 6, 8, and 10 weeks and 1, 2, and 4 weeks after the laboratory chow diet was resumed. This dose of virus was previously found to be 100% lethal (LD_{100}) for animals consuming the HL diet for 8 weeks. Four control animals feeding on laboratory chow were inoculated with virus at times corresponding to 0, 4, and 10 weeks on the HL diet. For additional controls, four mice consuming the HL diet for 4 and 10 weeks were inoculated with virus-free tissue culture media. Mortality was recorded daily for 2 weeks after virus inoculation.

Virus clearance studies. HC mice consuming the HL diet for 8 weeks and age-matched controls were inoculated i.p. with 3×10^8 PFU CV B5. At 15 min, 30 min, and 1, 2, 3, and 4 h after virus inoculation, peritoneal fluid, blood, and liver tissues were collected, and virus was quantitated in each by the plaque assay described above.

Antimicrobial activity to CV and *Listeria monocytogenes* in normal and in *Corynebacterium parvum*-treated mice. The ability of *C. parvum* to protect HC mice from a lethal dose of CV B5 was tested. HC mice

consuming the HL diet for 8 weeks were inoculated with 17.5 mg of *C. parvum* (Wellcome Research Laboratories, Beckenham, England) per kilogram of mouse, a dose previously found to enhance host resistance in this strain of mice (14). Six days later, these animals and untreated HC mice were challenged with 5×10^8 or 1×10^9 PFU of CV B5. Mortality was recorded over a 2-week period.

The comparative ability of *C. parvum* to protect chow-fed mice could not be assessed, as normal C57BL/6J mice are resistant to CV B5. Therefore, the above experiment was repeated by challenging *C. parvum*-treated HC and normal mice with *L. monocytogenes* and CV B4, both of which are lethal for normal and HC mice. *C. parvum*-treated and untreated mice from each dietary group were inoculated i.p. with an LD_{50} of *L. monocytogenes* (8.8×10^4 and 7.8×10^5 colony-forming units [CFU] for HC and normal mice, respectively) or intravenously (i.v.) with 1.0×10^5 , 5.0×10^6 , or 1.5×10^7 CFU of *L. monocytogenes*. Finally, *C. parvum*-treated and untreated mice from both dietary groups were infected i.p. with 1.7×10^3 PFU of CV B4. At the time of bacteria and virus inoculations, the liver and spleen of *C. parvum*-treated HC and normal mice were weighed and examined microscopically.

Inflammatory responses in normal and HC mice. The inflammatory responses of HC mice to a nonspecific stimulus were examined in two ways. The inflammatory agents thioglycolate and *C. parvum* were used to elicit a nonspecific inflammatory response in the peritoneal cavity. HC and normal mice were injected i.p. with 1.0 ml of a freshly prepared sterile 10% suspension of Brewer's thioglycolate (Difco Laboratories, Detroit, Mich.), or 17.5 mg of *C. parvum* per kilogram of mouse. At days 0, 1, 3, 5, and 7 after injection, peritoneum exudate cells were quantitated and differential cell counts were performed by staining the cells with Diff-Quik xanthene-thiazine (Harleco, Gibbstown, N.J.) and fluorescent acridine orange (1).

The inflammatory responses of HC and normal mice were also compared by the degree of footpad swelling in response to *C. parvum*. The right hind footpads of HC or normal mice were inoculated subcutaneously with 8 mg of *C. parvum* per kilogram of mouse in a volume of 0.03 ml. The left hind foot, serving as a control, was inoculated with sterile saline. At 1, 3, 7, and 14 days after inoculations, the *C. parvum*-injected foot and corresponding popliteal lymph nodes from mice in each dietary group were removed for histopathological sectioning. The footpad volume was calculated by the formula described by Attia and Weiss (2). Footpad swelling, expressed as the stimulation index, was calculated by the ratio of the stimulated footpad volume to the control footpad volume.

Antibody titrations. Serum neutralizing antibody to CV B5 was quantitated by the constant virus-varying serum technique in a microtiter (12), using HeLa cell monolayers. The 50% neutralizing endpoint then determined by the method of Reed and Muench (19), and antibody titers were expressed as the reciprocal of that serum dilution which protected 50% of the monolayers from viral cytolysis.

Hyperimmune serum. A high titer of virus-specific antisera was prepared by inoculation of C57BL/6J mice i.p. with 1×10^9 PFU CV B5 at weekly intervals for 4 weeks. The neutralizing antibody titer of the

TABLE 1. Plasma cholesterol values and enzyme activities in normal and HC mice^a

Mice	Time on diet/age (wk)	Cholesterol (mg/dl)			Enzyme activity (IU/liter) ^b	
		Free	Esters	Total ^c	ALP	ASP
Control	0/6	28.0 ± 1.6	61.0 ± 2.5	64.1 ± 2.6	237.4 ± 16.5	135.5 ± 2.7
	0/10	15.0 ± 2.8	56.3 ± 2.9	48.9 ± 4.4	125.1 ± 14.1	141.4 ± 17.7
	0/14	19.6 ± 0.9	76.3 ± 8.0	64.6 ± 5.0	—	—
	0/16	14.2 ± 2.2	56.2 ± 1.5	47.3 ± 2.6	87.7 ± 10.6	120.8 ± 25.5
	0/20	14.2 ± 3.8	87.5 ± 13.7	65.8 ± 4.3	104.5 ± 8.5	194.5 ± 30.6
HC	1/7	47.8 ± 4.9	97.2 ± 4.6	105.1 ± 6.5	142.1 ± 36.5	215.3 ± 54.8
	2/8	46.3 ± 2.7	60.2 ± 1.9	81.8 ± 3.8	184.8 ± 36.7	294.7 ± 73.5
	3/9	48.6 ± 1.4	108.9 ± 7.3	112.8 ± 5.6	102.6 ± 3.2	218.1 ± 44.5
	4/10	47.1 ± 1.4	96.8 ± 5.1	104.2 ± 2.2	121.3 ± 22.6	206.3 ± 20.4
	6/12	39.1 ± 7.9	88.3 ± 10.3	91.2 ± 13.9	128.8 ± 5.5	235.8 ± 36.8
	8/14	39.4 ± 3.5	134.7 ± 16.8	118.9 ± 12.8	179.2 ± 29.6	247.5 ± 35.3
	10/16	38.6 ± 5.8	112.8 ± 5.3	105.1 ± 6.9	141.9 ± 8.5	300.5 ± 91.8
	-1/17 ^d	33.4 ± 3.4	87.0 ± 7.1	84.3 ± 8.3	151.2 ± 9.7	241.7 ± 25.3
	-2/18 ^d	13.6 ± 2.4	74.3 ± 5.6	57.5 ± 4.5	154.9 ± 3.2	271.1 ± 10.2
	-4/20 ^d	22.5 ± 1.1	81.5 ± 3.7	70.5 ± 2.4	147.5 ± 8.5	235.8 ± 40.8

^a Values are means ± standard deviation of three plasma samples pooled from 15 animals.

^b ALP, Alkaline phosphatase; ASP, aspartate transaminase.

^c Total cholesterol is free cholesterol + 0.59 cholesterol esters.

^d Negative numbers indicate weeks on chow diet after the HL diet was consumed for 10 weeks.

pooled hyperimmune serum was 1.975 ± 137 U/1.0 ml. Normal mouse serum, obtained from noninfected mice in a similar manner, failed to neutralize 100 50% tissue culture infective doses of virus at a dilution of 1:20.

Passive antibody transfer. The ability of passively administered neutralizing antibody to protect HC mice from a lethal dose of CV was tested. HC and normal mice were inoculated i.p. with an LD₁₀₀ of CV B5. These same animals received 0.1 ml of hyperimmune antisera or normal mouse sera i.p. 2.5 h after virus infection. Additional HC and normal mice were inoculated with 0.1 ml of hyperimmune serum only. Mortality was recorded for each group over a 2-week period.

At 24 h after infection, three mice receiving virus and hyperimmune sera and 3 mice injected with hyperimmune sera alone were bled for antibody titrations. Plasma samples were incubated at 60°C for 1 h to inactivate CV before titrating the antisera as described above.

Statistical analyses. Statistical differences in the biological parameters between dietary treatments and among the time intervals measured were tested by the two-way analysis of variance. A correlation between virus-induced mortality and the biological parameters measured was tested by computing the correlation coefficient *r*.

Statistically significant differences in virus or bacteria-induced mortality between *C. parvum*-treated and untreated mice were calculated by chi-square analysis. Student's *t* test was used to measure significant differences in antibody titers and virus titers between the normal and the HC mice. In all cases, 5% ($P \leq 0.05$) was chosen as the level of significance.

RESULTS

Physiological parameters. Body and organ weights. Although the total body weight remained normal (23.6 ± 0.3 to 26.5 ± 1.19) in the

experimental group, the HL diet drastically increased the size of the livers. Liver weights of control, chow-fed mice remained constant ($5.8 \pm 0.4\%$ of body weight) through 10 weeks of age, but declined thereafter ($4.7 \pm 0.3\%$ of body weight), whereas liver weights of mice fed the HL diet rapidly increased ($7.3 \pm 0.8\%$ of body weight) beginning at 1 week to twice the normal value of 4.7 g at 10 weeks on the HL diet ($10.2 \pm 0.8\%$ of body weight). This dietary effect was reversible upon chow feeding, since liver weights of experimental mice decreased ($6.0 \pm 0.6\%$ of body weight) through 4 weeks after laboratory chow replaced the HL diet. There was no effect of the HL diet on spleen and thymus weights (0.4 ± 0.1 and $0.2 \pm 0.05\%$ of body weight, respectively).

Biochemical assays. Consumption of the HL diet led to a rapid increase in plasma-free cholesterol, cholesterol esters, and therefore total cholesterol in experimental animals (Table 1). Free cholesterol values doubled beginning at 1 week on the HL diet and remained at this elevated level throughout the 10 weeks. Likewise, cholesterol ester values and total cholesterol nearly doubled in mice fed the HL diet after 1 week. Continued consumption of the diet did not further increase the cholesterol levels but effectively maintained the plasma cholesterol concentration at twice the control values. Free, esterified, and total cholesterol levels returned to normal 2 weeks after chow feeding began. Plasma-free fatty acid, triglyceride, and protein values remained normal 19.4 ± 8.8 , 30.1 ± 16.1 , and 6.5 ± 0.9 mg/dl, respectively, in all experimental mice.

TABLE 2. Liver cholesterol and protein values in normal and HC mice^a

Mice	Time on diet/age (wk)	Cholesterol (mg/g)			Protein (mg/g)
		Free	Esters	Total ^b	
Control	0/6	3.2 ± 0.7	4.3 ± 0.2	5.8 ± 0.8	139.8 ± 4.7
	0/10	4.5 ± 0.3	2.3 ± 0.5	5.8 ± 0.4	123.7 ± 7.8
	0/16	2.6 ± 0.7	2.5 ± 0.6	4.1 ± 1.0	155.0 ± 22.5
	0/20	2.6 ± 0.5	0.6 ± 1.2	2.9 ± 0.1	159.5 ± 15.4
HC	1/7	4.2 ± 0.4	9.8 ± 1.4	9.8 ± 0.7	101.9 ± 9.9
	2/8	5.1 ± 1.3	4.0 ± 1.2	7.0 ± 1.3	126.2 ± 17.0
	3/9	5.1 ± 2.1	4.6 ± 1.2	7.8 ± 2.9	110.6 ± 10.9
	4/10	5.4 ± 0.5	4.9 ± 0.3	8.4 ± 0.4	146.9 ± 5.5
	6/12	7.2 ± 0.7	5.1 ± 0.4	10.2 ± 0.6	88.1 ± 8.0
	8/14	4.8 ± 0.7	4.8 ± 0.8	7.8 ± 1.1	74.0 ± 4.9
	10/16	5.4 ± 0.8	5.1 ± 0.3	8.5 ± 0.7	84.0 ± 4.3
	-1/17 ^c	5.9 ± 0.7	6.0 ± 1.1	9.4 ± 1.1	137.7 ± 2.9
	-2/18 ^c	4.0 ± 0.4	5.1 ± 0.5	7.0 ± 0.2	130.0 ± 17.8
	-4/20 ^c	3.2 ± 0.6	5.5 ± 0.7	6.6 ± 0.7	176.7 ± 10.4

^a Values are means ± standard deviation of determinations from four individual liver extracts.

^b Total cholesterol is free cholesterol + 0.59 cholesterol esters.

^c Negative numbers indicate weeks on chow diet after the HL diet was consumed for 10 weeks.

Plasma alkaline phosphatase activity decreased with age in the control group; however, the enzyme activity remained steady in the experimental group from 1 to 10 weeks on the HL diet (Table 1). Consequently, significant differences in alkaline phosphatase activity between the two dietary groups became evident with time. The enzyme activity was statistically elevated in the experimental mice at 8 and 10 weeks on the HL diet. Feeding laboratory chow did not lower the enzyme activity in the experimental group. Plasma aspartate transaminase activity nearly doubled in experimental mice in comparison to controls at all times from 1 to 10 weeks on the HL diet, and then through the 4 weeks of chow feeding (Table 1). However, there were no significant changes in the enzyme activity with time within each dietary group. Plasma bilirubin averaged 0.3 ± 0.1 IU/liter in both dietary groups.

Hepatic cholesterol concentrations changed significantly with age as evidenced in control animals and with the duration of feeding the HL diet (Table 2). Free cholesterol levels in the liver of chow-fed mice decreased by 50% after 10 weeks of age. Values in the experimental group, however, increased at 6 weeks on the diet (12 weeks of age) to three times the control value and remained at this elevated level in comparison to controls until 4 weeks after the resumption of chow feeding. Likewise, cholesterol ester and total cholesterol values in the livers of control mice decreased with age; in contrast, experimental mice showed a sharp increase in hepatic cholesterol beginning at 1 week on the HL diet. By 2 weeks, cholesterol ester and total cholesterol values decreased in the experimental

group; nonetheless, liver cholesterol values continued to rise thereafter to twice the control values and remained elevated when laboratory chow replaced the HL diet. In contrast to hepatic cholesterol, free fatty acid, and triglyceride values in the liver remained normal (4.2 ± 0.1 and 3.6 ± 0.2 mg/g, respectively).

The increase in hepatic cholesterol was accompanied by a significant decrease in liver protein per gram of tissue (Table 2). Hepatic protein levels were lower in HC mice in comparison to controls after 1 week and again at 6 through 10 weeks on the HL diet when total protein in the livers of experimental mice averaged one-half that of control values. However, this decrease occurred when the liver weights of HC mice were twice the normal value. The total amount of protein in the entire liver tissue of normal and HC mice was 728.5 and 848.4 mg of protein per gram of liver, respectively, at 16 weeks of age. Therefore, protein synthesis was not inhibited in the HC animals but the expansion to twice the liver weight, presumably due to cholesterol accumulation, effectively diluted hepatic protein levels to one-half of the normal concentration per gram of tissue. The reduction in protein per gram of liver was readily reversible, as protein values returned to normal within 1 week after chow feeding was resumed.

Blood cell counts. Erythrocyte and leukocyte cell counts were unaffected by consumption of the HL diet and averaged $9.9 (\pm 0.9) \times 10^6$ and $11.6 (\pm 3.6) \times 10^3$ cells per mm^3 , respectively, for both dietary groups.

Histopathological findings. The increase in hepatic cholesterol and concomitant decrease in liver protein per gram of tissue were associated

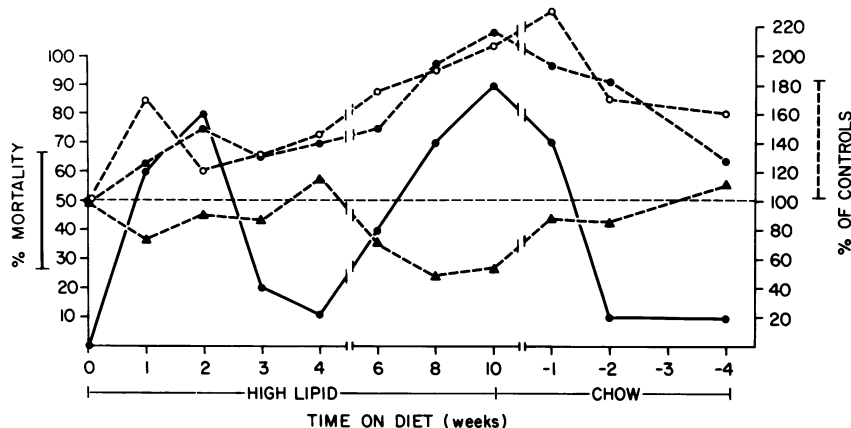


FIG. 1. CV-induced mortality in C57BL/6 mice. The solid line plots the changes in the percentage of mortality with time on the high lipid or return to chow (negative numbers) diet. The dashed lines plot changes in liver weight (●), total liver cholesterol (○), and liver protein (▲) with duration of the HL or chow diet.

with pronounced histopathological changes in hepatic morphology. As early as 1 week on the HL diet, fat vacuoles appeared in the periportal spaces of the liver. With longer exposure to the HL diet, the area of lipid vacuolization continued to spread outward from the periportal spaces and most of the hepatocytes were infiltrated with fat at 10 weeks on the diet. By 6 weeks on the diet, the fatty changes were accompanied by a loss of hepatic nuclei. There was generally no inflammatory response in the liver, although an occasional PMN was seen. When laboratory chow replaced the HL diet, the fatty changes in the liver were reversed progressively with time, and by 4 weeks on the chow diet, there was little or no fatty change in the livers. There were no histopathological changes in the spleen, thymus, or adrenal glands of all mice throughout the experiment. The cause of death in the virus-infected animals was most likely due to hepatic failure from hepatocyte destruction, since cytoplasmic necrosis and nuclear degeneration were obvious in livers from virus-infected HC mice.

Effects of dietary lipids on virus-induced mortality. Figure 1 shows the increase in virus-induced mortality with time on the HL diet. Mortality remained at a minimum through 4 weeks on the HL diet, but gradually increased thereafter to a peak of 90% after 10 weeks on the experimental feed. When HC animals were fed laboratory chow, virus-induced mortality declined and diminished to 10% after 2 weeks on the chow feed. There were no deaths among chow-fed mice inoculated with virus or among HC mice injected with control media at any time.

Although there were significant changes in several parameters as a result of the HL diet,

only those pertaining to the liver were statistically correlated with the increase in virus-induced mortality (Fig. 1). The increase in mortality from 4 to 10 weeks on the HL diet correlated significantly ($P \leq 0.05$) with increasing liver weight ($r = 0.94$) and increasing liver total cholesterol ($r = 0.96$) when liver cholesterol is expressed as a percentage of the control values. Mortality was also statistically correlated with decreasing liver protein per gram of tissue ($r = 0.833$) through 8 weeks on the HL diet. At the time of maximum susceptibility to CV, the liver weights were doubled in the experimental group ($10.2 \pm 0.8\%$ versus $4.7 \pm 0.4\%$ of body weight) and the total cholesterol concentration was also increased twofold (8.5 ± 0.7 versus 4.1 ± 1.0 mg/g of liver), whereas the total protein per gram of liver was reduced by one-half (84.0 ± 4.3 versus 155.0 ± 22.5 mg/g of liver). No statistical correlation was found between decreasing mortality, after HC mice were fed a chow diet, and any parameter measured.

Effects of metabolic alterations on immune functions. Virus clearance studies. The titers of CV B5 in the peritoneum, liver, and blood of normal and HC mice at various times after infection are shown in Fig. 2. Virus was cleared from the peritoneum at the same rate in both dietary groups, with approximately 10% of the intraperitoneal inoculum remaining after 15 min and 0.1% after 4 h. Significant differences, however, were seen in the rate of virus clearance from the liver and blood in the two dietary groups. Beginning at 1 h, virus titers in the livers of HC mice were more than a \log_{10} higher than controls and remained statistically elevated through 4 h. Likewise, the concentration of virus in the blood was 10-fold higher in the HC mice from 15 min through 2 h after infection.

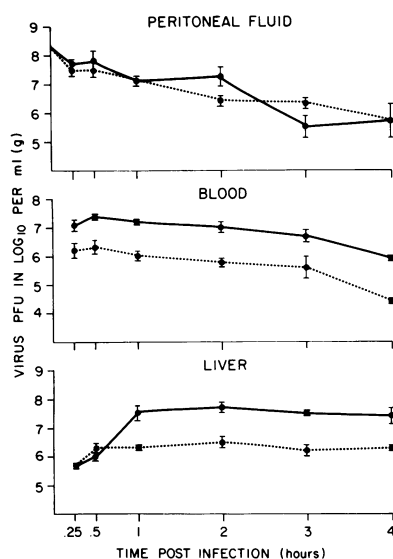


FIG. 2. Rate of clearance of CV B5 from the peritonea of C57BL/6 mice. The solid and dashed lines represent virus titers in HC and control animals, respectively, at various times after an i.p. inoculation of 3×10^8 PFU of CV B5. Brackets represent standard deviations among three individual mice.

Antimicrobial activity in normal and HC mice. Treatment with *C. parvum* before a CV B5 infection protected HC mice from a low dose (LD_{25}) of virus, but failed to protect these animals from an LD_{100} (Table 3). *C. parvum* was also ineffective in increasing the mean survival time of HC mice inoculated with either dose of virus. HC mice were more susceptible to CV B4

in comparison to chow-fed controls, since a dose of 2×10^3 PFU of CV B4 killed 50% of the normal untreated mice but 100% of the HC animals (Table 3). *C. parvum* treatment protected both normal and HC mice from an LD_{50} and LD_{100} of CV B4, respectively. As with CV B5, *C. parvum* failed to increase the mean survival time of HC mice infected with CV B4 as it did in the control animals.

The HC mice were also found to be more susceptible to *L. monocytogenes*, as evidenced by the differences in mortality from an i.p. injection of the bacteria (Table 3). A dose of 8×10^5 CFU administered i.p. to normal mice resulted in 50% mortality, whereas 10-fold less bacteria killed 100% of HC animals. However, *C. parvum* protected both dietary groups from an i.p. infection, since mortality was decreased and mean survival time increased in the treated animals. Similarly, *C. parvum* protected both dietary groups from an i.v. inoculation of 10^5 or 10^6 CFU of *L. monocytogenes* (Table 3). Nevertheless, differences in the protective capability of *C. parvum* in the normal and HC mice were revealed when a high dose (1.5×10^7 CFU) of bacterium was administered. Although *C. parvum* treatment reduced mortality from 100 to 57% and prolonged the mean survival time of normal mice infected with *L. monocytogenes*, it failed to protect HC animals from the high dose of bacteria both in terms of mortality and mean survival time.

The fact that *C. parvum* failed to induce hepatomegaly and splenomegaly and a dense cellular infiltrate in the liver and spleen of HC mice may in part explain the above-mentioned observation. At the time when normal *C. par-*

TABLE 3. Protective effect of *C. parvum* against CV and *L. monocytogenes* in mice fed a chow or HL diet

Inoculation	Diet	Dose (PFU or CFU)	Untreated mice		<i>C. parvum</i> -treated mice ^a	
			Mortality (%)	MST ^b (days)	Mortality (%)	MST (days)
CV B5	HL	5.0×10^8 i.p.	25 (2/8)	11.2 ± 5.3	11 (2/18) ^c	12.7 ± 3.7
		1.0×10^9 i.p.	100 (8/8)	2.0 ± 0	80 (8/10)	3.6 ± 2.0
CV B4	Chow	2.0×10^3 i.p.	50 (4/8)	10.7 ± 3.9	0 (0/18) ^c	14.0 ± 0
	HL	2.0×10^3 i.p.	100 (8/8)	5.7 ± 0.7	33 (6/18) ^c	8.1 ± 4.9
<i>L. monocytogenes</i>	Chow	8.0×10^5 i.p.	50 (4/8)	9.7 ± 4.6	5 (1/18) ^c	13.8 ± 0.5
		5.0×10^6 i.v.	100 (8/8)	3.0 ± 0	50 (4/8) ^c	9.3 ± 4.2
		1.5×10^7 i.v.	100 (8/8)	2.3 ± 0.5	57 (4/7) ^c	10.7 ± 3.7
	HL	8.0×10^4 i.p.	100 (8/8)	4.5 ± 0.7	11 (2/18) ^c	13.4 ± 1.8
		1.0×10^5 i.v.	100 (8/8)	3.2 ± 0.4	50 (4/18) ^c	11.0 ± 3.7
		1.5×10^7 i.v.	100 (7/7)	2.0 ± 0	87 (7/8)	5.5 ± 3.5

^a Mice received 17.5 mg of *C. parvum* per kg of mouse i.p. 6 days before bacteria or virus inoculations.

^b MST, Mean survival time (in days) \pm standard deviation. Experiment was terminated 14 days after bacteria or virus challenge.

^c Statistically significantly different from the untreated group by chi-square analysis.

TABLE 4. Effects of *C. parvum*^a on organ weights of normal and HC mice^b

Mice	Spleen		Liver	
	Wt (g)	Increase ^b	Wt (g)	Increase
Control ^d	0.3 ± 0.01	4.8 ± 0.1	3.3 ± 0.2	2.7 ± 0.2
HC ^c	0.1 ± 0.02	1.3 ± 0.2	2.7 ± 0.3	1.2 ± 0.1

^a *C. parvum* (17.5 mg/kg of mouse) was inoculated i.p. 6 days before removal of organs.

^b Values are means ± standard deviation.

^c The increase in weights was measured by calculating the ratios of organ weights from *C. parvum*-treated mice to the average organ weight of 15 untreated mice. Differences were significant at $P \leq 0.001$.

^d Values are an average of three mice fed laboratory chow.

^e Values are an average of five mice fed the HL diet.

vum-treated mice were infected with bacteria or virus, the livers and spleens of these chow-fed animals were three and five times the normal weight, respectively (Table 4). Focal areas of dense inflammatory cells could be seen in the liver, particularly in the periportal spaces (Fig. 3). Likewise, the follicular regions of the spleen were grossly enlarged 6 days after *C. parvum* treatment (Fig. 3). In contrast, the liver and spleen of HC mice treated with *C. parvum* did not increase significantly in weight by this time (Table 4). Furthermore, the inflammatory response seen in the livers and spleen of control mice were not evident in these HC animals (Fig. 3).

It appeared, therefore, that the inflammatory response was suppressed in the HC mice. For this reason, the response of peritoneal exudate cells to the stimulants thioglycolate and *C. parvum* were compared in normal and HC mice. The cellular responses to thioglycolate are depicted in Fig. 4. One day after thioglycolate stimulation, peritoneal monocytes, PMNs, lymphocytes, and therefore total cell counts were similar in the two dietary groups. However, a significant decrease in total cell counts was evident in HC mice from 3 to 7 days after thioglycolate inoculation in comparison to controls. This diminished response in the HC mice was due to a deficiency in PMN, monocytes, and lymphocytes at 3 and 5 days, and PMN and lymphocytes at day 7 after stimulation.

In contrast to thioglycolate, the cellular response to *C. parvum* was identical in the peritonea of normal and HC mice. At days 1 through 7 after *C. parvum* inoculation, the number of elicited monocytes, PMNs, and lymphocytes were similar in both dietary groups (peak of 1.2×10^7 cells per mouse at day 5). As in the peritoneum, *C. parvum* elicited comparable cellular responses in the footpads of normal and HC mice. The mean stimulation index for con-

trol mice reached a peak of 2.5 ± 2.2 at day 3 after stimulation; the index for HC mice reached a peak of 3.0 ± 0.9 at day 1 after injection of *C. parvum*. Likewise, there were no differences in the type and density of cellular infiltrate in histopathological sections of footpads and popliteal lymph nodes in both dietary groups.

Protection by passive antibody transfer. Table 5 summarizes the effect of passively administered antibody on virus-induced mortality. Intraperitoneal inoculations of hyperimmune sera were effective in boosting the serum-neutralizing antibody titers to CV B5, as evidenced by the serum antibody titers 24 h after administration of immune sera. Hyperimmune sera injected into HC mice 2.5 h after virus infection was effective in reducing mortality in the HC mice by 92%.

Kinetics of CV antibody formation. Table 6 summarizes the kinetics of the antibody response to CV B5 in normal and HC mice. Neutralizing antibody titers were not detectable until day 4 after infection in both dietary groups. Antibody synthesis was not impaired in the HC mice; furthermore, antibody titers were significantly higher at days 4 and 5 in the HC mice in comparison to controls. By day 7, antibody titers to CV B5 were not significantly different between the two dietary groups. Variations in antibody titers among individual animals resulted in large standard deviations, making biological evaluation of statistical differences difficult.

DISCUSSION

Although dietary-induced hypercholesterolemia resulted in numerous metabolic changes within the host, only an elevation in hepatic cholesterol correlated with decreased viral resistance. The elevation in hepatic cholesterol and increased liver weight resulted in a relative reduction in protein content per gram of tissue, even though the total amount of liver protein was not significantly reduced. A significant increase in mortality (50% or greater) occurred only when the hepatic cholesterol to protein ratio was 2.5. This effect held true for animals consuming the HL diet and after chow feeding was resumed. The absolute increase in hepatic cholesterol content at a constant protein level suggests that there may be a critical tissue cholesterol protein ratio at which impaired resistance becomes evident. Such a critical level was not evident when plasma rather than hepatic cholesterol/protein ratios were correlated with mortality.

The liver parenchymal and Kupffer cells are the major cells participating in the clearance of plasma lipid, with the net uptake of cholesterol from circulating low-density lipoproteins taking place only in the liver (7, 21). Evidence from the present studies suggest that consequently the

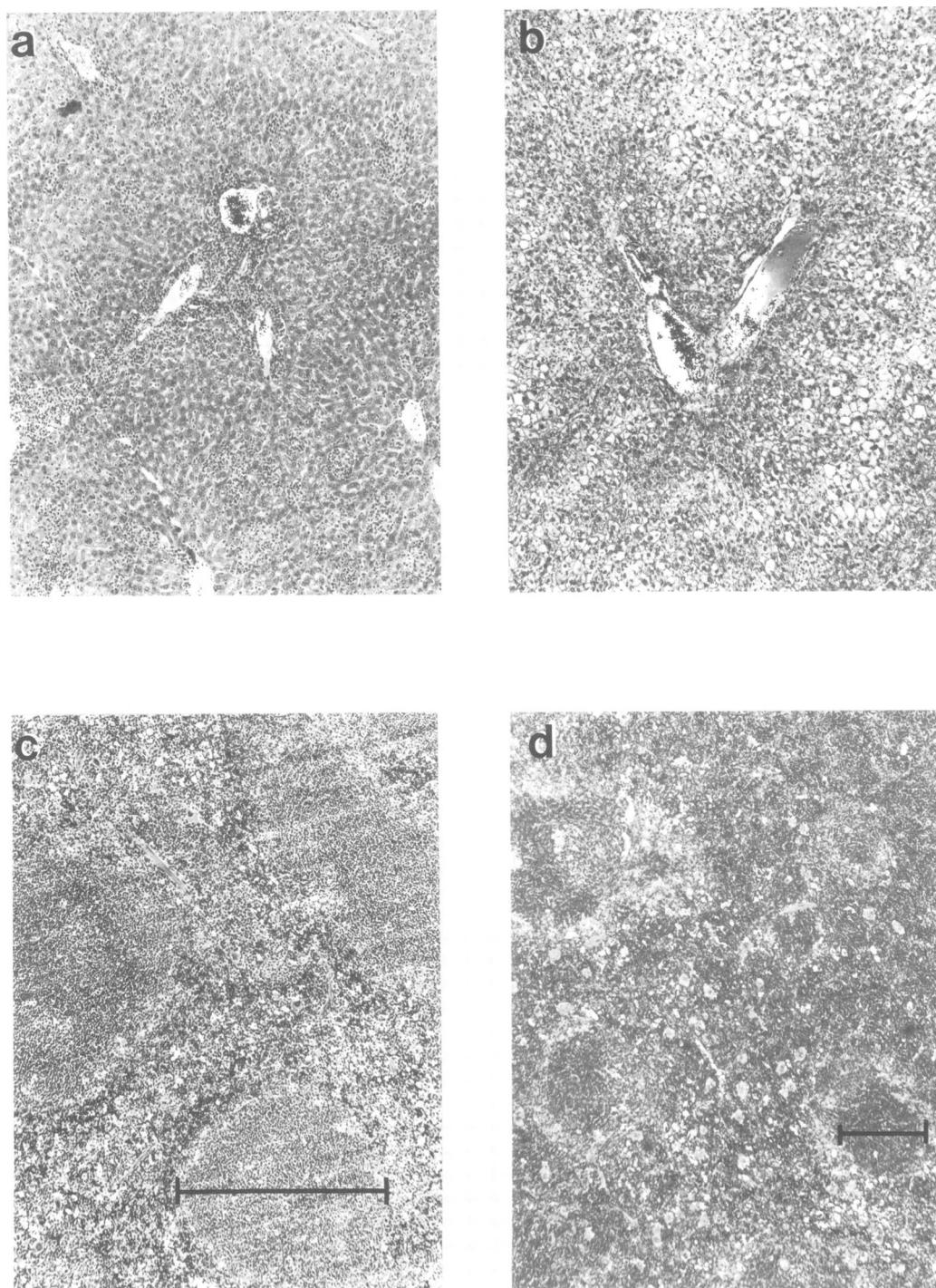


FIG. 3. Cellular infiltrate in the liver and spleen of *C. parvum*-treated C57BL/6 mice. Livers and spleens from normal (a and c) or HC (b and d) mice were examined on day 6 after treatment with 17.5 mg of *C. parvum* per kg of mouse. (a) The livers of normal mice were infiltrated with mononuclear cells and PMNs. The infiltrate was particularly dense in the periportal spaces. (b) In contrast, a dense cellular infiltrate was absent in the livers of HC mice. (c) In the spleen of normal animals, the follicles (shown by the bracket) became enlarged due to an influx of lymphoid cells. (d) However, a change in cell density was not evident in the follicles of HC. (All photographs, $\times 13.4$)

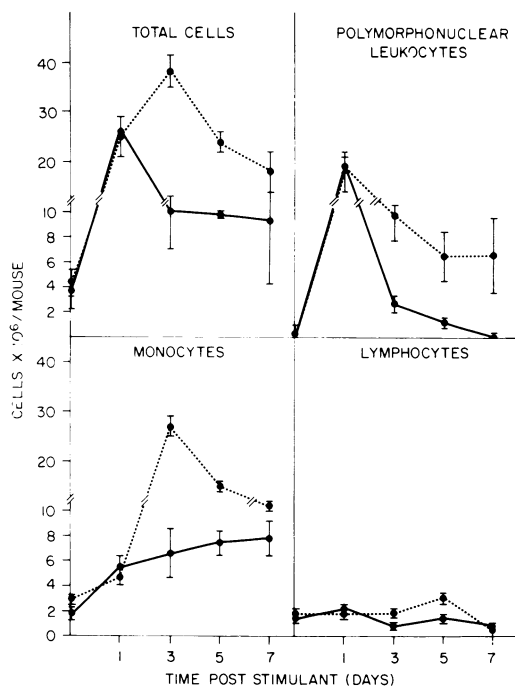


FIG. 4. Cellular response to thioglycolate in the peritonea of C57BL/6 mice. Mice were injected i.p. with 1.0 ml of 10% thioglycolate. The cellular response in the peritonea of HC and normal mice is depicted by the solid and dashed lines, respectively. Brackets represent standard deviations among three animals.

mononuclear phagocytic functions of cholesterol-laden livers are inhibited in hypercholesterolemia, a finding which supports previous studies using experimentally induced hypercholesterolemia (20). The decreased rate of virus clearance from the blood and liver of the HC animals indicates an impairment in the antiviral activity of Kupffer cells. In normal chow-fed animals, the virus titer in the liver increased by less than 1

TABLE 6. Antibody response to CV B5 in C57BL/6 mice fed the chow or HL diet

Days postinfection	Antibody titers ^a (U ^b /1.0 ml)	
	Chow diet	HL diet
1	<20	<20
2	<20	<20
3	<20	<20
4	28 ± 4	52 ± 12 ^c
5	100 ± 36	300 ± 142 ^c
7	288 ± 134	368 ± 22
14	6,860 ± 2,860	10,659 ± 2,860

^a Numbers represent the average antibody titer (± standard deviation) of three individual mice.

^b A unit is equal to that dilution of serum which neutralizes 50% of HeLa cell monolayers infected with 100 50% tissue culture infective doses of CV B5.

^c Significantly elevated compared with controls as determined by Student's *t* test.

log from 15 min through 4 h after infection, with no change in virus titers after 30 min. However, virus titers increased by 2 logs in the livers of HC mice during this time. Because a substantial amount of virus replication would not be expected during the first 4 h after infection (8), these data suggest a deficiency in the initial antiviral activity of fatty livers. The increased virus titers in the blood and liver of HC mice during this initial period of infection correlates with our previous finding that virus titers remain elevated in the blood and liver of HC mice in comparison to normal animals through a minimum of 1 week (4). In further support of decreased Kupffer cell functions in HC mice, these animals were found to be more susceptible to an intravenous inoculation of *L. monocytogenes*. *L. monocytogenes* administered intravenously rapidly localizes in the liver and spleen, which thereby become the key organs in early resistance to this bacterium (5).

TABLE 5. Effect of passively administered antibody on CV-induced mortality in normal and HC mice

Mice ^a	Virus (PFU per mouse)	Serum ^b	Antibody titer ^c	Mortality (%)
Control	None	Hyperimmune	ND ^d	0
Control	5 × 10 ⁸	Normal	ND	0
HC	None	Hyperimmune	126 ± 68 ^e	0
HC	5 × 10 ⁸	Normal	ND	100
HC	5 × 10 ⁸	Hyperimmune	96 ± 34 ^e	8 ^f

^a Each group contained six mice.

^b Mice inoculated with hyperimmune sera received 1.975 ± 687 U anti-CV B5-neutralizing antibody. Normal serum contained no detectable antibody to the virus. Animals were inoculated i.p. with serum 2.5 h after virus inoculations.

^c The antibody titer is expressed as U/1.0 ml of plasma. These titers were determined 24 h after the injection of serum.

^d ND, Not done.

^e Means ± standard deviation.

^f One out of 12 animals.

These observations suggest that excess dietary lipid in an otherwise nutritious diet may induce a hepatic blockade, an association which has not been previously demonstrated. However, evidence is also presented that the inhibitory effects of cholesterol extend beyond the Kupffer cells, since normal inflammatory responses are inhibited in HC Mice. Clearly, the monocytic and PMN responses to thioglycolate are inhibited in the peritonea of HC mice. The reason for the differing responses to thioglycolate and *C. parvum* in the peritonea is unknown, but perhaps the responses to each are elicited by different mechanisms. *C. parvum* administration is known to cause splenomegaly, hepatomegaly, macrophage granuloma, and an increase in the number of liver macrophages which originate from the bone marrow (9, 11, 13). A significant increase in liver and spleen weights with dense cellular infiltrates in these organs was evident in normal chow-fed mice. However, the liver and spleen weights of HC animals did not significantly change with *C. parvum* treatment and the cellular infiltrate was sparse, thereby associating excessive dietary cholesterol with a suppression of the inflammatory response. This may also explain in part the inability of *C. parvum* to protect HC mice from high doses of *L. monocytogenes* and CV.

Our observations that the cellular responses to *C. parvum* in the footpads of normal and HC mice were similar, although Kos et al. (14) showed that the inflammatory response to *C. parvum* injected into a tumor mass in the footpad of HC mice was abrogated, expand this finding and suggest that the type of antigenic challenge may be relevant. This is further supported by our present observations on the difference in response to *L. monocytogenes*, CV B4 and CV B5. Finally, the dose of challenging agent was relevant, since we could induce protection with *C. parvum* against low doses of the *L. monocytogenes*, CV B4, and CV B5, but not against high doses of the same agents.

Although passively administered neutralizing antibody was found to be effective in protecting HC mice from a lethal CV infection, antibody does not appear to have a major role in natural resistance to this virus. The HC mice which are susceptible to CV B5 and their highly resistant controls developed comparable antibody titers to CV B5. These results are in agreement with those of Woodruff, who showed that inhibition of mononuclear inflammatory cell functions decrease resistance to CV B3 without altering the neutralizing antibody response (23).

This report demonstrates the inhibitory effect of dietary-induced hypercholesterolemia on nonspecific immune responses. The criteria by which immunosuppression was assessed in

these experiments were focused on mononuclear inflammatory cell functions and antibody responses because these are known to be of primary importance in resistance to CV. However, further analysis of the immunocompetence of the HC mice may reveal additional dysfunctions in cellular immune reactions. Such dietary-induced immunosuppression may have broad implications in resistance to numerous infectious diseases and to tumorigenesis. These studies suggest that dietary ratios may exert selective effects on the immune system and raises the possibility that such alterations may be a useful tool in modulating immunity.

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