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Inhibition of Host Resistance by Nutritional Hypercholesteremia

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Previous experiments showed that nutritionally induced hypercholesteremia in mice caused an increase in susceptibility to coxsackievirus B, with a marked suppression of cellular infiltrates in infected tissues and an increased mortality. The present studies demonstrated that a hypercholesteremic diet was associated with an inhibition in host resistance as measured by susceptibility to *Listeria monocytogenes* infection and the growth of two transplanted syngeneic murine tumors. Moreover, the ability of *Corynebacterium parvum* to induce regression of a transplanted methylcholanthrene-induced fibrosarcoma was inhibited in hypercholesteremic hosts, as was the histiocytic infiltration normally accompanying *C. parvum* inoculation. In contrast, the peritoneal macrophages from *C. parvum*-treated hypercholesteremic mice were indistinguishable from similarly treated macrophages from normal mice with respect to their in vitro tumoricidal activity and the presence of a cell surface antigen associated with activated macrophages. Hypercholesteremia was also associated with a decreased antibody response to sheep erythrocytes in vivo, but did not appear to exert a detrimental effect on B- or T-cell blastogenesis when tested in vitro. The findings that the hypercholesteremic diet was associated with an impairment in the host immune response and increased susceptibility to viral, bacterial, and tumor cell challenge are discussed with respect to virus-lipid interactions in the pathogenesis of atherogenesis and diabetes mellitus.

Hyperlipemia, particularly hypercholesteremia, has long been recognized as one of the independent risk factors associated with atherosclerosis (35, 39) as well as being a complication of diabetes mellitus (5, 27, 28). Previous studies demonstrated that a hypercholesteremic (HCHOL) diet significantly augmented the susceptibility of outbred mice to coxsackievirus B infection and induced a more severe and divergent pathology than observed in coxsackievirus B-infected normal mice (6, 31). Histopathological examination of tissue sections from these animals revealed that the marked inflammatory infiltrate which normally occurred in coxsackievirus infection was conspicuously absent in HCHOL mice, suggesting a diet-mediated immunological impairment (31). Observation that resistance to coxsackievirus B3 is dependent on the function of the reticuloendothelial system (42, 43), whereas lipids may act as modulators of this system's function, are consistent with these findings (8, 12).

Dietary fats have been shown to increase the incidence of 7,12-dimethyl-benz-α-anthracene- (21) or diethylstilbesterol-induced (13) mammary tumors as well as increasing the number of eye, ear duct, and liver tumors in animals fed high-fat diets and treated with 2-acetylaminofluorene (14). Although the mechanism(s) by which high-fat diets promote tumor growth is unknown, suppression of the immune system may be in part related to this effect. Both saturated (palmitic and stearic) and unsaturated (oleic, linoleic, and arachidonic) fatty acids have been shown to inhibit lymphocyte blastogenesis (34). Moreover, Chapman and Hibbs (8) have demonstrated inhibition of in vitro macrophage-mediated tumor cell cytotoxicity by a low-density lipoprotein-like fraction of human and mouse sera as well as by artificial enrichment of plasma membranes with cholesterol. Consequently, the present studies were designed to evaluate the effect of nutritionally induced hypercholesteremia on host resistance in the mouse. The results of this study are consistent with nutritionally mediated inhibition of host resistance as a consequence of a diet rich in animal fat cholesterol, cholic acid, and sucrose.

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MATERIALS AND METHODS

Animals. Male C57BL/6J inbred mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and outbred Crl:(ICR)BR(CD-1) male mice were obtained from Charles River, Wilmington, Mass.; upon averaging 22 g in body weight, the mice were placed on the HCHOL diet (6, 30, 31). Experiments were initiated after 7 months on the diet.

HCHOL diet. The HCHOL diet was originally developed for outbred CD-1 mice (31). Inbred C56BL/6J mice, however, failed to thrive on this regimen, and the diet had to be diluted with 25% laboratory mouse chow (6). Animals gained weight, and a significant elevation in plasma total cholesterol levels, similar to the increase in outbred CD-1 mice, was obtained (6). Consequently, the HCHOL diet was modified to contain 1% cholesterol, 0.5% cholic acid, 18% lard, 18.5% casein, 52.6% sucrose, 5% cellulose, 4% salts (Hegstead), 0.2% choline chloride, and 0.1% vitamin mixture described previously (31). These modifications amount to an increase in protein and a decrease in cholesterol, cholic acid, and lard equivalent to the effect obtained by the dilution of the original diet (31) with 25% laboratory mouse chow (Ralston Purina Co., St. Louis, Mo.). The control diet contained 50.8% nitrogen-free extract which is mostly carbohydrates, 4.5% fats, 23% crude protein, 6% fiber, and 8.5% minerals. Daily food consumption of animals on either diet was equivalent (31); however the HCHOL diet caloric content was significantly higher than that of the control diet.

Plasma protein levels of C56BL/6J mice on the HCHOL diet and on normal laboratory chow were measured by the procedure of Lowry et al. (32) and did not differ significantly, 6.7 ± 0.2 g/100 ml versus 6.8 ± 0.5 g/100 ml, respectively. Animals on the HCHOL diet had 15 to 20% lower body weight than normally fed mice. Plasma lipoprotein electrophoresis demonstrated a significant hyperbetalipoproteinemia, with a significant decrease in alpha-globulin and prealbumin levels (Table 1).

Lipid determinations. Plasma total cholesterol, cholesterol esters, free fatty acids, and triglycerides were determined by using a modification of a thin-layer chromatography procedure described previously (30, 31). Blood for lipid determinations was collected from the tail vein in 0.1-ml tubes after an overnight fast. After centrifugation, 0.002 ml of plasma was applied directly to a glass plate coated with a 0.5-mm layer of Silica Gel G (Camag Co., New Berlin, Wis.). The chromatogram was first developed in chloroform-methanol-water-acetic acid (65:25:4:1) to 5 cm, dried, and developed successively to 5, 10, and 19 cm, respectively, with hexane-diethyl ether-acetic acid (70:30:1) developer. The plates were then sprayed with 50% H_2SO_4 and charred at 220°C for 45 min. The charred chromatogram was scanned on a recording densitometer with an electronic integrator (Corning Glass Works, 750 model, Corning, N.Y.). The amount of each lipid constituent was calculated by reference to an internal standard in each sample and a reference lipid mixture on the same plate.

TABLE 1. *Effect of diet on lipoprotein levels in C57BL/6J mice*[a]

Diet	α	β	Prealbumin
HCHOL	10 ± 3	79 ± 6	12 ± 4
Lab chow	19 ± 10	27 ± 6	55 ± 12

[a] According to the procedure of Noble (40), values are in percentage of total lipoproteins, are based on three to six animals each, and are presented as mean ± standard deviation.

Plaque assay for IgM-producing spleen cells. Spleen cells were collected from normal and HCHOL mice 4 days after an intravenous (i.v.) injection of 5 × 10^8 sheep erythrocytes (SRBC). Spleen cells secreting 19S immunoglobulin M (IgM) antibody against SRBC were enumerated by a modification (23) of the hemolytic plaque assay in liquid originally described by Cunningham and Szenberg (10).

In vitro lymphocyte blastogenesis. The T and B blastogenic responses of lymphoid cells were determined by using phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, Mich., or Burroughs-Wellcome, Research Triangle Park, N.C.) and *Escherichia coli* lipopolysaccharide (LPS) (O111.B4, Difco), respectively, in a modification (2) of the culture technique originally described by Hartzman et al. (19). Blastogenesis was measured by [^{3}H]thymidine incorporation into deoxyribonucleic acid in a microtiter assay system.

Listeria monocytogenes infection. *L. monocytogenes* (ATCC 9303) was kindly provided by Page Morahan of the Department of Microbiology, Medical College of Virginia. Serial 10-fold dilutions were made in sterile phosphate-buffered saline, and groups of eight normal or HCHOL mice were injected i.v. in the tail vein with doses ranging from 6.4 × 10^3 to 6.4 × 10^7 colony-forming units (CFU) in 0.2 ml. Cells were plated to determine the viable titer at the time of inoculation. Mice were checked twice daily, and deaths were recorded for a period of 14 days. The 50% lethal dose (LD_{50}) endpoint was calculated according to the method of Litchfield and Wilcoxon for dose effect experiments (29).

Growth of transplanted tumors. Tumor growth experiments were performed as previously described (18). Briefly, groups of normal or HCHOL mice were challenged in one hind footpad with either 5 × 10^4, 2 × 10^5, or 5 × 10^5 cells of a syngeneic methylcholanthrene-induced fibrosarcoma (MCA 2182) or the Lewis lung carcinoma (LLC) in 0.05 ml. Three days after tumor challenge, half of the mice in each group were injected intralesionally (i.l.) with 17.5 mg of a heat-killed vaccine of *Corynebacterium parvum* (Burroughs-Wellcome) per kg of body weight. Mice were checked daily for death or tumor appearance for a period of 60 days. Tumor dimensions were recorded twice weekly, and tumor volumes were calculated as previously described (1). The mean survival time (MST) and the percentage of tumors regressing in each group were calculated. Tumor-bearing mice were counted as regressors if no tumor was present 90 days after *C. parvum* inoculation.

In vitro cytotoxicity. Activated macrophages were collected by peritoneal lavage with Hanks balanced salt solutions (HBSS) from normal and HCHOL mice which had received an intraperitoneal (i.p.) injection of 17.5 mg of *C. parvum* per kg 7 days earlier.

Control peritoneal macrophages were obtained from mice which had been injected with saline. Target cells consisted of secondary mouse embryo fibroblast (MEF) cells and cultured LLC cells. Peritoneal exudate cells (PEC) were adjusted to 1×10^5 to 2×10^6 cells/ml, and 0.5 ml was pipetted into the 81-mm^2 chamber of one eight-chambered slide (Lab Tek, Naperville, Ill.) Slides were incubated for 2 h at 37°C in 5% CO_2–95% air and washed four times with sterile 0.15 M NaCl to remove nonadherent cells. The adherent macrophages were overlaid with 5×10^4 LLC or MEF cells, incubated for 60 h, stained, and scored as previously described (26). Macrophage monolayers were generally >97% macrophages as judged by esterase staining and phagocytosis of latex particles. Macrophage-to-tumor cell ratios of 20:1, 10:1, 5:1, and 0:1 were used. The cytotoxicity scores of 0, 1+, 2+, 3+, and 4+ represented 0 to 10%, 11 to 25%, 26 to 50%, 51 to 75%, and 76 to 100% cytotoxicity, respectively. The cytotoxicity scores of duplicate chambers were added and divided by 8, the highest cytotoxicity score possible, and multiplied by 100 to give the percent cytotoxicity.

Detection of an antigen associated with activated macrophages. PEC from normal and HCHOL mice were assessed for an activation-specific surface antigen by indirect immunofluorescence as previously described by Kaplan and Mohanakumar (24). Cells were collected 7 days after *C. parvum* or saline injection and washed three times in HBSS. Portions of cells (4×10^6 cells in 0.2 ml) were incubated for 30 min on ice with 50 μl of an appropriately absorbed rabbit anti-mouse macrophage or rabbit anti-mouse activated macrophage antiserum, washed three times in HBSS, and then incubated with 50 μl of a 1:2 dilution of fluorescein-conjugated goat anti-rabbit IgG $F(ab)_2$. After 30 min on ice, the cells were washed as before, suspended in phosphate-buffered saline containing 10% glycerol, and examined for fluorescence. The percentage of fluorescent cells was calculated from a minimum of 100 cells.

Histopathology of transplanted tumors. Normal and HCHOL mice were challenged with 5×10^4 MCA 2182 cells and treated with *C. parvum* i.l. as described above. At 4, 8, 12, and 16 days after *C. parvum* treatment, two mice from each group were sacrified. Tumor-bearing feet were removed just above the metacarpals and cut in half longitudinally before fixation overnight in Bouin fixative (33). After fixation, all tissues were washed overnight in gently running water. The feet were decalcified for 12 h by the formic acid-sodium citrate method before embedding. Tissue sections from paraffin-embedded samples were prepared at 5-μm thickness, stained with Harris hematoxylin and eosin, and then examined and photographed with a Wild M20 microscope. Kodak Panatomic-X film, ASA 32, and a Wratten 11 filter were used to optimize soft photographic contrast.

RESULTS

Plasma total cholesterol levels after 7 months on the diet were approximately 346 ± 60 mg/100 ml as compared with 119 ± 6 mg/100 ml in male C57BL/6J mice fed the control diet. These values are in agreement with our previous observation (6), and no significant elevation in either plasma-free fatty acids or triglyceride levels was observed.

Effect of hypercholesteremia on susceptibility to *L. monocytogenes*. Our previous experiments indicated that an HCHOL diet diluted with 25% lab chow increased the susceptibility of inbred C57BL/6 mice to both the short-term lethal effects and long-term pathogenic effects of coxsackievirus B5 (6). Therefore, it was important to determine whether the effects of an HCHOL diet were unique to this virus or if an HCHOL diet induced a general change in host resistance to viral, bacterial, and tumor growth. Consequently, mice were injected with *L. monocytogenes* to determine if susceptibility to a bacterial agent was increased in the HCHOL host. Groups of 10 mice each, fed either an HCHOL diet or a control diet, were inoculated with 6.4×10^3 to 6.4×10^7 CFU of *L. monocytogenes* at log intervals. The LD_{50} was reduced from 1.5×10^7 CFU in normal mice to 3.7×10^5 CFU in HCHOL mice, a greater than 40-fold increase in susceptibility (Table 2).

Effect of HCHOL on growth of transplanted tumors. A third parameter of host resistance in addition to viral and bacterial resistance is tumor growth. To elucidate further the resistance of mice on an HCHOL diet versus mice on the control diet, we determined the capacity of mice to resist the growth of two syngeneic tumors. A preliminary experiment compared the MST of control, chow-fed mice with that of HCHOL mice after inoculation with either LLC or MCA 2182. In both cases, survival times were significantly reduced in the HCHOL mice compared with chow-fed controls (Table 3). Similarly, the tumor growth rate was increased in the HCHOL mice compared with chow-fed controls (data not presented).

Previous investigators have implicated the macrophage as an effector cell in *C. parvum*-induced tumor regression as well as suggesting that cholesterol could modulate the cytotoxic effect of macrophages toward tumor cells (18).

TABLE 2. *Effects of L. monocytogenes infection in normal and HCHOL C57BL/6 mice*[a]

Nutritional status of host	LD_{50} (CFU/mouse)	Potency ratio[b]	95% confidence limits (CFU/mouse)
Normal	1.5×10^7	40.3	5.3×10^6–4.1×10^7
HCHOL	3.7×10^5		9.5×10^4–1.4×10^6

[a] Eight normal or HCHOL mice were used for each dose of *L. monocytogenes* from 6.4×10^3 to 6.4×10^7 CFU at log intervals.

[b] Potency ratio = LD_{50} of normal mice/LD_{50} of HCHOL mice.

Therefore, mice were inoculated in the footpad with 5×10^4 MCA 2182 cells and 3 days later injected i.l. with 17.5 mg of *C. parvum* per kg. Fifty percent of the normal mice receiving MCA 2182 cells which were inoculated with *C. parvum* on day 3 underwent tumor regression, whereas *C. parvum* failed to protect HCHOL mice from tumor growth (Table 4) and death. Although no tumor regressions were noted in normal or HCHOL mice inoculated i.l. with 2×10^5 MCA 2182 tumor cells and *C. parvum*, there was a significant increase in the MST of normal mice given *C. parvum* (MST = 37.2 ± 4.5 days) versus the HCHOL mice given *C. parvum* (MST = 25.6 ± 3.1 days) ($P < 0.05$).

Histological evaluation of MCA 2182 tumors with or without i.l. *C. parvum* in normal and HCHOL mice. At 4 days, the tumors of both diet groups appeared as a thin layer of tumor cells, perhaps 50 to 60 cell diameters thick, situated between the dermis of the footpad and the ligaments passing to the toes. At this interval, there was only a moderate leukocytic infiltration in the paratumor connective tissues. The tumor mass in both the normal and HCHOL diet groups progressively enlarged through day 16; however, their cellular compositions became widely divergent with respect to host leukocytes appearing at the tumor site. In the tumor-bearing normal mice treated with *C. parvum*, inflammatory cells at the tumor site increased progressively in tissue concentration at days 8, 12, and 16. Polymorphonuclear cells (PMN) and mononuclear cells (largely histiocytes) appeared first in the connective tissue around the tumor nodule (Fig. 1A). At days 12 and 16, foci of PMN were present within the tumor mass (Fig. 1B) and were usually associated with regions of necrosis. Additionally, numerous histiocytes were present in localized regions of the tumor mass (Fig. 1B). The latter cells had large euchromatic nuclei that were usually oval in form and had a thin peripheral ring of heterochromatin and frequent nucleoli.

TABLE 3. *Effect of hypercholesteremia on survival of mice inoculated with the LLC or the MCA 2182 cells*

Tumor	Nutritional status of host	Tumor cell dose	MST[a]	P
LL	Normal	5×10^4 ($n = 10$)	36.8 ± 2.7	<0.05
	HCHOL		28.6 ± 2.6	
MCA 2182	Normal	5×10^4-5×10^5[b] ($n = 30$)	26.8 ± 5.5	<0.05
	HCHOL		22.8 ± 1.4	

[a] Days ± standard error.

[b] Groups of 10 C57BL/6J mice each inoculated with 5×10^4, 2×10^5, or 5×10^5 MCA 2182 tumor cells were pooled for presentation.

TABLE 4. *Effect of hypercholesteremia on C. parvum-mediated tumor regression*[a]

Nutritional status of host	No. of regressions/no. of mice challenged	% of mice protected from tumor growth by *C. parvum*
Normal	0/10	50[b]
Normal + *C. parvum*	5/10	
HCHOL	1/10	0
HCHOL + *C. parvum*	1/10	

[a] C57BL/6J mice were injected with 5×10^4 MCA 2182 tumor cells, followed 3 days later by an i.l. injection of 17.5 mg of *C. parvum* per kg.

[b] $P < 0.05$. The chi-square test with the Yates correction factor was used.

The histopathological picture seen in tumor-bearing HCHOL mice treated with *C. parvum* varied substantially from that seen in the normal group. At days 12 and 16, moderate numbers of leukocytes, principally lymphocytes, were present in the paratumor connective tissue (Fig. 1C). The tumor mass was a very densely packed population of sarcoma cells with numerous mitotic figures (Fig. 1D). In contrast to the pronounced inflammatory infiltrate and necrosis in normal tumor-bearing mice treated with *C. parvum*, the HCHOL tumor-bearing mice had no foci of necrosis and essentially no leukocytes were present within the tumors. Only a few PMN were present, particularly at days 8 and 12, in the connective tissue around the tumor, and none were present within the tumor. Although a few histiocytes were present in the connective tissue, lymphocytes were the principal leukocyte present. Interestingly, these lymphocytes were the most dense in the dermis just above the tumor, and in this region there was a narrow zone of tumor cells that contained very few mitotic figures and cells with somewhat hyalinized appearing cytoplasm. Despite the pronounced change in the histiocytic inflammatory infiltrate of HCHOL mice compared with controls, there was not a significant change in the number of peripheral monocytes in these animals. Differential leukocyte counts in the HCHOL mice indicated 65.7 ± 3.0% lymphocytes, 27.8 ± 3.0% neutrophils, and 6.5 ± 0.8% monocytes versus 86.4 ± 2.7% lymphocytes, 9.3 ± 2.6% neutrophils, and 4.3 ± 0.9% monocytes in control animals.

Effect of hypercholesteremia on *C. parvum* activation of macrophages. HCHOL mice, in contrast to normal animals, were unable to resist the growth of transplanted tumors in spite of i.l. *C. parvum* treatment, and previous experiments had demonstrated that *C. parvum*-induced tumor regression was related to macro-

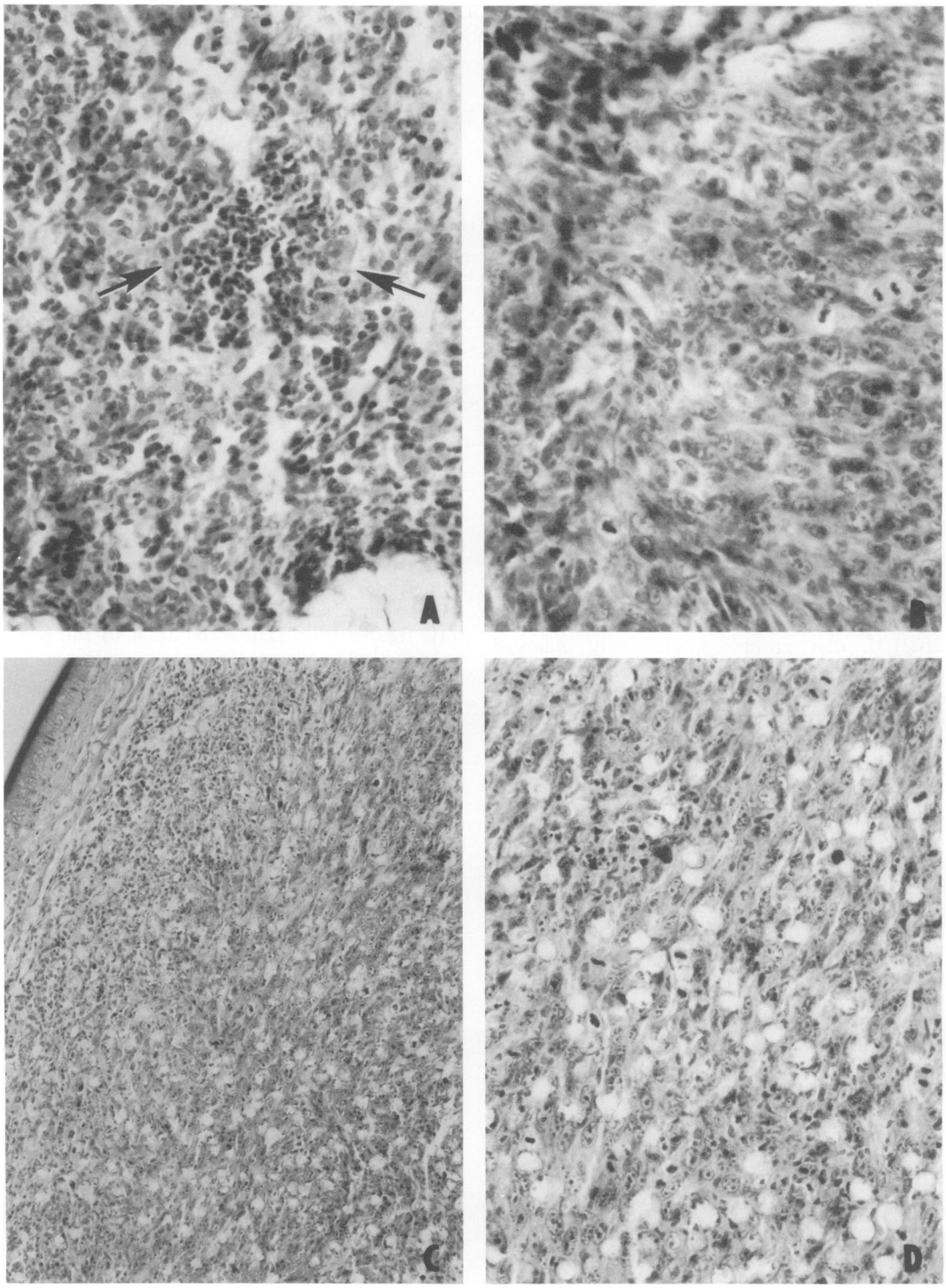

FIG. 1. *Micrographs from C56BL/6 mice bearing footpad MCA 2182 fibrosarcomas that had been treated with C. parvum 12 days previously. The animals had been maintained on a control diet (A, B) or a high-fat diet (C, D). All fields were stained with hematoxylin and eosin. (A) Extensive inflammatory cell (PMN [arrows] and histiocyte) infiltration of the hypodermis and tumor mass. ×400. (B) Histiocytes infiltrate the tumor mass. ×400. (C) Very modest infiltration of inflammatory cells in the connective tissue between the tumor mass and the epidermis. ×200. (D) Only a few leukocytes are present in the tumor mass. Numerous mitotic figures are present. Note disposition of fat throughout the tumor mass. ×400.*

phage activation (18). It therefore was important to determine if macrophages could be activated by *C. parvum* in HCHOL mice. Two criteria of macrophage activation, the expression of a new cell surface antigen associated with activation (22) and the cytotoxic activity against tumor but not normal cells in vitro, were evaluated.

The membrane antigen specifically associated with *C. parvum*-activated macrophages could be detected on macrophages from both normal and HCHOL mice after i.p. inoculation of *C. parvum* (Table 5). In contrast, control PEC from HCHOL or normal mice were fluorescent only when stained with macrophage-specific antisera and not with antisera specific for activated macrophages. The percentage of control PEC that stained was equivalent to background staining established by using normal (nonimmune) rabbit serum. The activation-specific antigen was not found on nonactivated PEC from saline-injected mice. By this criterion, macrophages of HCHOL mice could be activated by injection of *C. parvum*.

PEC from normal and HCHOL mice treated i.p. with *C. parvum* were found to be equally effective in killing tumor cells in vitro at PEC-to-tumor cell ratios as low as 5:1 (Table 6). Minimal background cytotoxicity was detectable with control PEC from normal animals, but only at the 20:1 PEC-tumor cell ratio. Neither activated nor control PEC exhibited cytotoxicity against the normal (nontumor) MEF cells, as has previously been reported (1). It is clear from these data that *C. parvum* administration to HCHOL mice resulted in activation of macrophages at least as detected by their capacity to function in vitro.

TABLE 5. *Detection of an antigen associated with activated macrophages on PEC from either HCHOL or normal C57BL/6J mice inoculated with C. parvum*

Nutritional status of PEC donor	Inducing agent	% Positive cells with given primary rabbit antiserum[a]		
		Anti-macrophage	Anti-activated macrophage	Normal
Normal	*C. parvum*	67	66	6
	Saline	41	4	5.5
HCHOL	*C. parvum*	44	48	6
	Saline	52	5	0

[a] Immunofluorescent activity of normal rabbit serum, anti-macrophage serum, and anti-activated macrophage serum was tested in an indirect immunofluorescence assay on PEC from mice 7 days after inoculation with 17.5 mg of *C. parvum* per kg or 0.15 M NaCl. The specificity of these sera has been previously described (4, 33).

Effect of hypercholesteremia on manifestations of humoral immunity and blastogenesis. The possibility that hypercholesteremia could be altering B- and T-cell activities was investigated. The B-cell activity in HCHOL mice was assessed by enumerating the spleen cells producing 19S antibody to SRBC in vivo and by LPS-induced blastogenesis in vitro. Significantly fewer antibody-producing cells were present in spleens of HCHOL mice than in normal mice (Table 7). However, no difference in blastogenesis as measured by [^{3}H]thymidine incorporation in response to LPS was found with either diet group of C57BL/6 mice (Table 8). However, spleen cells from HCHOL outbred CD-1 mice incorporated a significantly greater amount of radioactivity than did cells from normal CD-1 mice.

The effect of the HCHOL diet on T-cell function was examined by phytohemagglutinin-induced blastogenesis. No difference in the response of spleen cells from HCHOL or normal mice was found in either strain (Table 8).

DISCUSSION

Loria et al. (31) and Campbell et al. (6) have previously shown that HCHOL mice are more susceptible to viral infection than the normal counterparts. This observation was extended in this report to include decreased resistance to bacterial infection and to tumor growth, while implicating the macrophage as one impaired cellular element of host resistance. Our data were obtained by inducing hypercholesteremia in

TABLE 6. *Effect of hypercholesteremia on in vitro tumor cell cytotoxicity by PEC from HCHOL and normal C57BL/6J mice*

Nutritional status of PEC donor	Inducing agent[a]	% Cytotoxicity at various macrophage/target cell ratios[b]							
		0:1		5:1		10:1		20:1	
		LLC	MEF	LLC	MEF	LLC	MEF	LLC	MEF
Normal	*C. parvum*	0[b]	0	87	0	100	0	100	0
	Saline	0	0	0	0	0	0	13	0
HCHOL	*C. parvum*	0	0	87	0	100	0	100	0
	Saline	0	0	0	0	0	0	0	0

[a] PEC were used 7 days after i.p. inoculation of 17.5 mg of *C. parvum* per kg or 0.15 M NaCl.
[b] Calculated as described in Materials and Methods.

TABLE 7. *Effect of hypercholesteremia on the antibody response to SRBC in C57BL/6 mice*

Nutritional status of spleen cell donor	Antibody-producing cells/10^6 spleen cells ± SE[a]
Normal	294 ± 34.6[b]
HCHOL	182 ± 21.6

[a] The number of antibody-producing cells was assayed 4 days after an i.v. injection of 5×10^8 SRBC. Data represent the average of eight mice/group. SE, Standard error.

[b] $P < 0.001$.

mice by nutritional means and are in agreement with results reported by DiLuizo (12) where lipids injected i.v. also adversely influenced the reticuloendothelial system and the immune response. Our in vivo observation on the effects of *C. parvum* on tumor growth are consistent with the in vitro observation by Chapman and Hibbs (8) that lipid, probably cholesterol, can inhibit macrophage-mediated tumor cell killing.

Woodruff and Kilbourne (48) reported that severe undernutrition in the form of marasmus resulted in a 60% reduction in body weight and a drastic increase in susceptibility to coxsackievirus B3. However, graded undernutrition, which reduced body weight by only 34%, did not cause such an effect. Restoration of marasmic mice to normal food intake on the day of virus challenge resulted in no deaths 6 days later and the loss of virus susceptibility.

The lower body weights of the animals on the HCHOL diet was not the result of a caloric restriction or less intake but the result of an imbalance of nutrients due to overnutrition. Even though the weight reduction of the HCHOL mice was less marked than that of the animals on the graded undernutrition, susceptibility to coxsackievirus B5 was significantly increased.

Finally, increased susceptibility to coxsackievirus B5 has recently been detected in HCHOL animals with normal body weights (A. E. Campbell, R. M. Loria, G. E. Madge, and A. M. Kaplan, unpublished data). These results are also in good agreement with the observations of Fiser et al. (16) which showed that monkeys fed a high-fat, high-cholesterol diet had impaired development of precipitating antibodies to ovalbumin-enhanced susceptibility to tuberculin antigen and an increased rate of clearance of colloidal carbon from blood. These observations strongly suggest that the data presented in this communication are not due to the lack of sufficient nutrition in the HCHOL mice but rather related to the lipid abnormalities induced by the diet.

Hypercholesteremia resulted in a decreased antibody response to SRBC in vivo, but did not appear to exert a detrimental effect on B- or T-cell blastogenesis when tested in vitro. In inbred C57BL/6 mice, incorporation of [^{3}H]thymidine in response to LPS did not reveal any diet-mediated impairment of B-cell blastogenesis. Thus, the reduction in the number of antibody-producing spleen cells in HCHOL mice may be due to diminished uptake and processing of antigen by macrophages or to a direct effect of lipids on B or T cells, or both, in vivo. Spleen cells from outbred HCHOL CD-1 mice responded to LPS with an enhanced incorporation of radioactivity compared with normal animals. The basis for this observed difference with cells of CD-1 and C57BL/6 mice is unknown. This may be a reflection of a general change in membrane lipid composition in B cells of the CD-1 strain, resulting in more efficient interaction with this mitogen. Similar discrepancies between the in vivo and in vitro immunological response were also reported by Fernandes et al. (15) for the diabetic mutant C57BL/Ks db+/db+ mouse. When compared with nondiabetic db+/m+ and +m/+m mice, the diabetic mutant demonstrated markedly altered in vivo immune responses characterized by a significantly diminished capacity to reject allogeneic skin grafts and generate cytotoxic T cells after sensitization with EL-4 lymphoma cells and an increase in the antibody response to SRBC. In contrast, spleen cells from db+/db+ mice demonstrated only minimal alterations in in vitro responses to mitogens and allogeneic cells and no alteration in their in vitro plaque-forming cell response (15). These discrepancies observed between in vivo and in vitro immunological assays suggest that the alterations in in vivo immune function

TABLE 8. *Effect of hypercholesteremia on the response of C57BL/6 and CD-1 spleen cells to phytohemagglutinin (PHA) and LPS*[a]

Mouse strain	Nutritional status of spleen cell donor	Response (cpm ± SE)	
		LPS	PHA
C57BL/6	Normal	58,667 ± 1,756	129,500 ± 8,197
	HCHOL	77,667 ± 10,202	147,000 ± 14,414
CD-1	Normal	33,167 ± 11,812[b]	75,667 ± 35,201
	HCHOL	98,333 ± 10,104	81,333 ± 28,872

[a] Peak response to the entire dose-response curve for PHA or LPS for spleen cells of normal and HCHOL mice. The PHA dose response was carried out at dilutions of 1:5, 1:10, 1:20, and 1:40, with the peak response occurring at 1:10 or 1:20 in all animals. The LPS dose response was done at 1, 5, 30, and 60 μl per well, with the peak response occurring at 5 or 30 μg. The background cpm varied from 400 to 11,000 in the absence of mitogen. SE, Standard error.

[b] $P < 0.01$ (normal compared with HCHOL).

of db+/db+ mice may be a consequence of the abnormal metabolic environment present in these animals. Similarly, the nutritional hypercholesteremia obtained in our animals could induce metabolic changes which would result in an impairment in immune function in vivo.

Upon i.v. injection, *L. monocytogenes* localizes in liver and spleen macrophages. North (41) and Cheers et al. (9) have shown that resistance to this organism depends upon the innate bactericidal or bacteriostatic activity of nonimmune macrophages followed by the acquisition of cell-mediated immunity. Data presented here do not permit us to distinguish between these two mechanisms. However, there are other factors in the HCHOL host that suggest macrophage impairment. Gross histopathological examination showed livers twice the normal size which were friable and yellowish in color. Histological examination revealed hepatocytes filled with lipid vacuoles, with severe fatty changes in the liver (6, 31, 32). Furthermore, up to 65% of the monocytes and 48% of the neutrophils in peripheral blood were found to contain lipid vacuoles by oil red O staining (L. J. Sniczek and R. M. Loria, unpublished data). These morphological changes may indicate functional differences as well. Improper or inefficient presentation of antigen by macrophages could indirectly result in diminished cell-mediated immunity.

Further evaluation of macrophage activities supported the suggestion that macrophages were involved in diet-mediated impairment of the immune response. *C. parvum* has been shown to induce resistance to the growth of transplanted tumors by activating macrophages to tumoricidal capacity (25). *C. parvum* was unable to induce tumor regression in HCHOL mice at a tumor cell dose which resulted in tumor regression in 50% of the normal mice treated with *C. parvum*. At a higher dose of 2×10^5 tumor cells, no regression was observed in either group of animals, though a significant prolongation of survival was observed in normal but not HCHOL mice.

The ineffectiveness of *C. parvum* in inducing tumor resistance in HCHOL mice was not due to a lack of macrophage activation. A membrane antigen associated with *C. parvum*-activated macrophages was detected on the surface of PEC from both normal and HCHOL mice that had received *C. parvum*. Furthermore, *C. parvum*-activated PEC from HCHOL mice were found to be as effective as those from normal mice in their ability to kill tumor cells in vitro. Histopathology demonstrated that the HCHOL animals suffered an impairment in the number of inflammatory cells at the tumor site after *C. parvum* treatment. Even though some leukocytes, primarily lymphocytes, and some histiocytes, but essentially no PMN, were present in the dermis above the tumor, the migration of leukocytes into the tumor mass was severely inhibited. In contrast, a significant inflammatory response consisting principally of histiocytes occurred in the normal tumor-bearing animals that were treated with *C. parvum*. This picture was similar to that previously reported for animals bearing LLC that were given pyran copolymer (45). This inability of i.l. *C. parvum* to effectively focus macrophages in the tumor may have been due to either an impaired chemotactic response in the macrophages or an impairment in the afferent or efferent ability of lymphocytes to produce chemotactic factors.

The findings that an HCHOL diet associated impairment in host immune response was associated with an increased susceptibility to viral, bacterial, and tumor cell challenge are of particular relevance to studies of atherogenesis and diabetes mellitus. The results are consistent with the hypothesis that group B coxsackieviruses, which have been reported to replicate, localize, and persist in the arterial blood vessel (6), could induce in the HCHOL host an aberrant immunological response, resulting in further vascular injury and atherosclerosis (6, 17, 20, 37). Similarly, impairment of the host immune response would also favor the establishment and propagation of monoclonal cells according to Benditt's hypothesis on atherosclerosis (3, 4).

The diabetic host has been known for its increased susceptibility to pyogenic bacterial infections (7, 44, 46, 49) and its predisposition to hypercholesteremia which, independently, may influence the pathogenicity of pyogenic bacteria (11). Furthermore, an increase in susceptibility to viral infection and autoimmune mechanisms have also been implicated as etiological factors in diabetes mellitus (38, 47). Additional studies are currently underway to analyze the effects of hyperlipidemia on the individual cell subpopulations involved in various parameters of host resistance as they relate to the present findings as well as to their potential immunopathogenic role.

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