Altered Immune Functions in Hypercholesterolemic Monkeys

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Humoral and cellular immune responses to several antigens were compared in control and hypercholesterolemic groups of monkeys. Chronic hypercholesterolemia, with concomitant hyperphospholipidemia and hypotriglyceridemia, was produced experimentally by feeding monkeys a high-fat, high-cholesterol diet. When studied prior to infection, hypercholesterolemic monkeys exhibited impaired development of precipitating antibodies against ovalbumin, enhanced sensitivity to tuberculin antigen (stimulated apparently by mycobacterial components in complete Freund adjuvant), and an increased rate of clearance of colloidal carbon from blood. During pneumococcal infection the ability of neutrophils from hypercholesterolemic monkeys to reduce nitroblue tetrazolium dye showed an increase greater than that of control monkeys; both groups exhibited increased but comparable final clearance rates of colloidal carbon, although the increment of increase was smaller in hypercholesterolemic monkeys.

A deficiency of dietary nutrients is generally believed to reduce the competence of a host to resist infectious illness. In contrast, it is not widely recognized that host resistance against experimentally induced infections may also be diminished by the presence of obesity or by the long-term ingestion of a diet containing abnormally high quantities of fat (2). Such a conclusion has been reached by investigators who studied either bacterial or viral infections in various species of laboratory animals (10, 13, 19) and in overweight infants (17). However, the mechanisms by which a lipid excess might be detrimental to the host have not been elucidated. The present study describes the occurrence of altered humoral and cellular immune functions in rhesus monkeys that had chronic diet-induced hypercholesterolemia and other related plasma lipid abnormalities.

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MATERIALS AND METHODS

Induction of hypercholesterolemia in monkeys.

A total of 16 healthy, well-conditioned *Macaca mulatta* were studied. Eight monkeys were given a cholesterologenic diet as described by Armstrong et al. (1). This diet provided 44% of calories as carbohydrate, 15% as protein, and 41% as fat (including both crystalline and egg yolk cholesterol). An equal number of control monkeys, matched by weight and sex, were fed an equicaloric diet of identical appearance and flavor which contained 76% of calories as carbohydrate, 20% as protein, and 4% as fat (8).

No between-group differences in caloric intake or body weight were observed during the 7-month experimental period (8). An earlier publication described alterations in lipid metabolism of monkeys on the high fat diet (8). These differences included significantly higher plasma cholesterol concentrations (335% of control group values), significantly higher plasma phospholipid concentrations (155% of the control group average), and significantly lower triglyceride concentrations (40% of control) (8). Plasma-free fatty acid values, total serum proteins, and immunoglobulin G, M, and A concentrations did not differ appreciably between groups (8).

Immunological studies. After hypercholesterolemia developed, all monkeys were inoculated subcutaneously with 15 mg of crystalline ovalbumin dissolved in physiological saline and emulsified in complete Freund adjuvant (Difco). A subcutaneous

booster dose of 15 mg of ovalbumin in physiological saline was administered 3 weeks later. Serum samples were obtained at weekly intervals to assess hemagglutinin and precipitin antibody responses. Sheep erythrocytes coupled to ovalbumin with bis-diazotized benzidine were employed for hemagglutinin titrations (11). Ouchterlony gel diffusion techniques were used for precipitin titrations and identity studies. Precipitin reactions with twofold serial dilutions of serum, and ovalbumin concentrations of 1, 10, 100, and 1,000 $\mu\text{g}/\text{ml}$ were evaluated. Sera that reacted at dilutions $>1:8$ with the highest concentration of antigen were considered to have high titers.

The possible inhibitory effect of high serum lipid on the precipitin reaction was investigated by performing parallel titrations with monkey antiovalbumin sera that were diluted with an equal volume of preimmune serum from control or hypercholesterolemic monkeys. Induction of cutaneous sensitivity to ovalbumin was investigated 3 months after the booster injection; 0.1-ml volumes containing 5 μg of ovalbumin or appropriate control solutions were inoculated intradermally. Sites were observed for the presence of immediate, early, or delayed dermal responses.

In accordance with procedures established for routine care of monkeys in our laboratories, skin test on the eyelid with 0.1 ml of tuberculin intradermic (Haver-Lockhart, Shawnee, Kan.) was applied to monkeys on arrival and at monthly intervals thereafter.

Two months after the primary inoculation with ovalbumin emulsified in complete Freund adjuvant all monkeys were injected intracutaneously at different sites on the shaved abdomen with 0.1-ml volumes of several skin test antigens, including intermediate and second U.S.P. purified protein derivative (PPD), Dermatophyton O (Hollister-Stier Laboratories, Spokane, Wash.), Trichophyton (Hollister-Stier Laboratories, Spokane, Wash.), Streptokinase-Streptodornase (Lederle Laboratories, Pearl River, N.Y.), and physiologic saline as a control.

The ability of monkeys to clear colloidal carbon from their blood was studied by the method of Stabb et al. (15) to assess reticuloendothelial system (RES) activity.

Studies during pneumococcal infection. After base-line biochemical and immunological studies were completed, all monkeys in both groups were infected experimentally by intravenous inoculation with 10^8 type I virulent *Diplococcus pneumoniae* (8). Within 12 h, all monkeys appeared ill and exhibited fever. By 24 h after inoculation, body temperatures ranged from 102.5 to 104 C, pneumococcal bacteremia was present, and white blood counts of all monkeys were more than double the corresponding preinjection values (8). Plasma cholesterol and phospholipid values in both groups exhibited a significant decrease as compared with preinoculation values (8); in contrast, triglyceride concentrations increased in hypercholesterolemic monkeys and declined in control monkeys (8).

Reduction of nitroblue tetrazolium (NBT) dye by neutrophils collected 24 h after inoculation was studied by the histochemical method of Park et al.

(14). Concurrently, RES function was estimated by determining colloidal carbon clearance rates (15). All monkeys were killed 27 h after inoculation to provide tissues for studies of cholesterologenesis (8). Necropsy was performed immediately; complete histopathological studies were supplemented by detailed attempts to detect the presence of tubercle bacilli by culture and staining techniques.

RESULTS

Although the development of hemagglutinating antibodies to ovalbumin was similar in both groups, hypercholesterolemic monkeys exhibited an altered progression of precipitin antibody responses (see Fig. 1). High-titer ($>1:8$) precipitin antibodies developed in seven of eight control monkeys; all showed precipitin bands against more than one component of ovalbumin. In contrast, no hypercholesterolemic monkey developed any high-titer precipitin antibodies. By 6 weeks, only four hypercholesterolemic monkeys showed low-titer precipitin antibodies. By 14 weeks only three showed two precipitin bands; these differences were significant ($P < 0.05$ and < 0.01). Demonstration of precipitins apparently was unaffected by the alterations in serum lipids; at optimal antigen concentrations, reactions of identity were demonstrated between positive sera from hypercholesterolemic and control monkeys. Moreover, addition of preimmune hypercholesterolemic serum to precipitin-positive sera from either group did not alter the appearance or titer of positive reactions.

Three months after ovalbumin booster injections all monkeys were skin tested on the abdomen with 5 μg of ovalbumin. Arthus reactions appeared within 4 h in five of eight control

FIG. 1. Sequential development of precipitating antibodies against components of ovalbumin in control (circles) and hypercholesterolemic (squares) monkeys. The arrows indicate times of primary, booster, and skin test injections. High-titer ($>1:8$) samples from each monkey are shown by solid symbols. The number of bands of precipitating reactions are shown on the ordinate.

monkeys and three of seven hypercholesterolemic monkeys. A delayed positive reaction, with induration of 10 mm in diameter at 24 and 48 h, occurred in six control and five hypercholesterolemic monkeys.

Routine skin tests to tuberculin were consistently negative in all monkeys throughout the early phases of this study. However, five monkeys with hypercholesterolemia developed a positive reaction to second-strength PPD of greater than 10-mm induration at 48 h when tested 2 months after immunization with ovalbumin in complete Freund adjuvant. Three of the same monkeys also had positive responses to intermediate-strength PPD. No evidence of tuberculosis could be found during subsequent clinical and roentgenographic studies conducted in these monkeys over a 2-month period. All other monkeys remained tuberculin negative, and no monkey in either group exhibited an abnormal response to any of the other antigens used in the skin test battery.

Clearance of colloidal carbon from blood of noninfected monkeys was assessed with additional groups of hypercholesterolemic or control monkeys to avoid affecting the RES capacity of the experimental groups. In these noninfected monkeys, the hypercholesterolemic group was found to have significantly higher clearance rates than the corresponding control group (Fig. 2). Twenty-four hours after inoculation with pneumococci, clearance rates for colloidal carbon in both experimental groups increased significantly above the respective noninfected group base-line values. Although the magnitude of increase was small in hypercholesterolemic monkeys (Fig. 2), both infected groups achieved comparable final clearance values.

In neutrophil suspensions prepared from infected control monkeys 24 h after pneumococcal infection, the mean incidence of cells capable of reducing NBT dye was 13%. A significantly higher value, 24%, was observed in suspension from the infected hypercholesterolemic monkeys ($P < 0.05$). The incidence of dye reduction by neutrophilic leukocytes from uninfected normal or hypercholesterolemic rhesus monkeys is 3 to 4%.

No evidence of localized infection or between-group differences in pathology existed 27 h after inoculation with pneumococci. Atheromatous lesions were absent in both groups, no pathological changes could be identified in lymph nodes, RES tissues, or bone marrow, and there was no evidence of a tuberculous infection in any tuberculin-positive or -negative monkey. In the hypercholesterolemic monkeys, no direct correlation was observed between alterations of lipid

concentrations and alterations of immune functions.

Chronic, naturally acquired parasitic infestations were present to an equal degree in monkeys of both groups; all showed scattered pulmonary mite lesions due to *Pneumonyssus simicola*, two monkeys from each group carried the intestinal worm *Oesophagostomum apiosomum*, and one monkey in each group had a parasite-induced polyp of the gastrointestinal tract.

DISCUSSION

In these studies, experimentally induced hypercholesterolemia in monkeys appeared to be associated with atypical humoral and cellular immune responses. Although the present investigations were exploratory in nature and provided only limited information about specific capabilities, the findings considered in their entirety suggested distinct differences in patterns of immunological responsiveness for hypercholesterolemic and control monkeys. These differences take on added significance in view of the relatively modest plasma lipid abnormalities, the absence of atheromatous lesions in blood vessels, and the absence of foamy macrophages in lymph nodes.

These studies thus provide the first experimental evidence in primates that altered immune responses are associated with a dietary intake high in fat. An alteration in immune mechanisms or a decrease in respiratory function was proposed by Tracey et al. (17) in accounting for the greater incidence of viral illness in overweight infants and children. Although respiratory functions were not measured in the present study, differences between groups seem unlikely in view of comparable body weights and progression of clinical illness.

A few scattered reports in early literature describe alterations of immune phenomena or phagocytosis that may follow the experimental induction of hypercholesterolemia (2). Chassin and Bruger (3) suggested in 1939 that agglutinin responses to typhoid antigens were slightly enhanced in rabbits given a high-cholesterol diet. In contrast, hemagglutinin responses to ovalbumin in our hypercholesterolemic monkeys were unaffected, whereas precipitin responses were diminished. Although precipitin titration techniques are not as sensitive as hemagglutination, they did show clear-cut differences between groups at times when precipitins were generally present in control monkeys and generally absent in the hypercholesterolemic group. Later, after additional hypercholesterolemic mon-

FIG. 2. Clearance of colloidal carbon from blood of monkeys, shown as mean $\pm$ 95% confidence limits for each group. All values for each monkey were converted to a percentage of its zero time, 100% value, to allow calculation of group means. Statistical significance of differences between groups were shown by *t* tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.002$).

keys had developed precipitin antibodies, Arthus reactions which require such antibodies were relatively comparable when both groups were compared. Our hypercholesterolemic monkeys developed positive delayed skin responses to tuberculin after receiving Freund adjuvant, whereas control monkeys did not. The positive tuberculin reactions in hypercholesterolemic monkeys seemed to suggest increased sensitivity to the mycobacterial components incorporated in complete Freund adjuvant, since the possibility of active tuberculosis was not detected by extensive clinical, radiological, and histological examinations or by culture. The development of delayed hypersensitivity to ovalbumin indicated that both dietary groups were capable of mounting an adequate cellular response. Differences between groups in response to tuberculin or ovalbumin could have been due to the type or amount of antigen required to stimulate T-lymphocytes or to differences in antigen processing by macrophages or lymphocytes.

This possible relationship between cholesterol and immune functions might also explain altered depressed responses to vaccines during a viral illness. We previously reported enhanced cholesterol utilization in both man and rhesus monkey during experimental viral and bacterial infections (8, 9, 12). Although at present it is highly speculative, increased cholesterol utilization and degradation possibly mediated through

the RES (4) might alter ability of the host to take up and process antigens.

DiLuzio (5) demonstrated in 1960 that the RES was involved in the clearance of lipids from blood. However, he found no significant impairment of RES function during brief periods of alimentary lipemia in dogs. Stuart and Davidson (16) reported that intravenous injection of 30 mg of cholesterol oleate into mice causes depression of RES function and that antibody formation was reduced if antigen was given during the period of RES depression. Subsequently, DiLuzio and Woolees (6) found that intravenous injection of milligram quantities of methyl palmitate into mice delayed RES clearance of colloidal carbon for many days and depressed the immunological response to particulate antigens.

In contrast to the effects observed after introduction of pharmacologic quantities of lipids into the blood stream, changes in RES function induced in our hypercholesterolemic monkeys by long-term dietary manipulation indicated that the ability of the RES to clear colloidal carbon was enhanced. An increased rate of RES clearance, reported by Wagner et al. (18) to occur during bacterial infections of man, was observed during pneumococcal infection of monkeys. During the infection the response of control monkeys was sufficiently greater than that of hypercholesterolemic monkeys to result in comparable final clearance rates of colloidal

carbon in both groups. This rate may represent the maximum capability for RES clearance in the monkey under conditions of this study.

Increased capacity of neutrophils to reduce NBT dye, characteristic of bacterial infections in man (7, 14), was observed in both groups of monkeys with pneumococcal infection. Although the greater number of NBT-positive cells in hypercholesterolemic monkeys could be interpreted to indicate a more vigorous host defensive reaction, Feigin et al. (7) showed that the patients with partially treated bacterial infections exhibited greater NBT dye responses than those whose infections were treated effectively.

Concurrent lipid studies (8) necessitated tissue measurements 27 h after monkeys were inoculated with pneumococci. Consequently observed differences in immune responsiveness could not be related to a change in host resistance or to survival. However, altered immune function could contribute to the impaired host resistance that is said to be associated with an excess of body fat or dietary lipid intake.

A sizeable portion of the American population has an overt or unrecognized derangement of body lipid metabolism, i.e., a familial or acquired abnormality that may range from simple obesity to a complex lipid dysfunction associated with abnormal enzyme actions or transport system defects. It is not known if any of these abnormalities would increase the susceptibility of an individual to infection or alter the responsiveness to vaccines. Our findings in nonhuman primates suggest that such questions should be asked, and that an effort should be made to evaluate the magnitude and importance of these relationships in man.

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