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Research Article

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Mechanisms Responsible for Defective Human T-Lymphocyte Sheep Erythrocyte Rosette Function Associated with Hepatitis B Virus Infections

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ABSTRACT The expression of selected lymphocyte surface-membrane markers was evaluated in 37 patients with acute viral hepatitis B, 10 of whom were studied serially through the resolving and convalescent phases of disease. Bone marrow-derived (B) lymphocytes were identified by reference to surface immunoglobulin, whereas normal thymus-derived (T) lymphocytes were assayed by their capacity to form spontaneous nonimmune rosettes with sheep erythrocytes (E rosettes, ER). During the acute and resolving phases of viral hepatitis B, the relative and absolute number of ER-positive lymphocytes was significantly reduced, whereas the number of surface immunoglobulin-positive lymphocytes and the absolute lymphocyte count remained normal. This resulted in the appearance of a third population of cells, deficient in respect to both surface immunoglobulin and ER markers. Such cells accounted for nearly 25% of peripheral blood lymphocytes, approximately 5×10^6 ml blood.

Depression of the number of ER-positive lymphocytes occurred at least once during the course of disease in every patient studied serially, and was observed in 55 of 67 individual assays of the 37 cases of acute viral hepatitis B. Lymphocytes from some patients reacquired ER function when cultured in fetal calf serum but not in the presence of autologous serum. Such autologous serum was capable of suppressing ER function of lymphocytes from normal donors. The extrinsic suppression of ER function by a serum factor (designated as the Rosette Inhibitory Factor), was found to be time dependent, characterized by a 4-h latent period and requiring approximately 18 h for maximum attenuation of ER function.

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The Serum Rosette Inhibitory Factor was: (a) heat and freeze-thaw stable, (b) nondialyzable, (c) physically separable from hepatitis B surface antigen, (d) not a lymphocytotoxic antibody, and (e) had the buoyant density of a lipoprotein. This extrinsic mechanism was observed in 41.8% of patients with reduced numbers of ER-positive lymphocytes.

The Rosette Inhibitory Factor was not detectable in the serum of the remaining 58.2% of the cases of acute and resolving viral hepatitis B despite the presence of reduced numbers of ER-positive lymphocytes. The lymphocytes from these cases did not reacquire ER function when cultured in the absence of autologous serum. The mechanisms responsible for the suppression of normal ER function in these cases appears to be intrinsic to the lymphocytes and not the result of a humoral factor. The T lymphocyte lineage of cells deficient in respect to ER function, whether of intrinsic or extrinsic type, was demonstrated by their capacity to form spontaneous rosettes with neuraminidase-treated sheep erythrocytes.

Both intrinsic and extrinsic suppression of T lymphocyte ER function commonly occurred during the first 4 wk of acute viral hepatitis B. 9 of the 10 patients followed serially continued to manifest defective ER function at 12 wk. However, the intrinsic defect was observable only in those patients whose liver disease had resolved or was resolving, whereas the extrinsic defect was observed only in two patients with biochemical and histological evidence of unresolved hepatitis at that time.

INTRODUCTION

Human thymus-derived (T) lymphocytes, the principal effectors of cellular immunity, form nonimmune rosettes

(E rosettes, ER)¹ with sheep erythrocytes and lack readily demonstrable high-density surface-membrane immunoglobulin (SIg)(1). The converse is true of bone marrow-derived (B) lymphocytes, which are primarily involved in the humoral antibody response. These two types of cell-surface markers have served as primary probes for the identification of B and T lymphocytes and facilitated investigation of the cellular biology of the lymphoid system.

T-lymphocyte ER formation is an active cellular function attributable to a surface-membrane receptor (2), and is dependent upon protein and nucleic acid synthesis (3). It is modulated by alterations in intracellular levels of cyclic nucleotides (4); and it has been found by some to parallel certain other parameters of T-lymphocyte function including phytohemagglutinin responsiveness and delayed cutaneous hypersensitivity responses in selected studies of primary immunodeficiency states (5). The expression of ER function may serve as an index of the functional integrity of T lymphocytes, since it is defective in many disease states thought to be associated with altered or deficient cellular immune responsiveness such as malignancy (6), systemic lupus erythematosus (7), and viral hepatitis (8).

The hepatitis B virus has been established as the etiologic agent responsible for long-incubation hepatitis, now known as viral hepatitis B. It is clear, however, that only a small minority of infected individuals develop clinical evidence of disease. When clinically apparent, infection may be manifest as: (a) acute self-limited viral hepatitis; (b) rarely as fulminant hepatocellular necrosis; (c) persistent infection associated with mild or severe chronic hepatic injury; or (d) as a carrier state with variable liver function. In these settings, hepatitis B virus-associated surface (HBsAg) and core (HBcAg) antigens may be demonstrable in hepatocytes (9). Indeed, a similar spectrum of disease states has been induced in chimpanzees after inoculation with a single pool of HBsAg-positive plasma (10). Thus, in hepatitis B virus infection, other factors besides the presence of virus appear to play a critical role in determining the character of the host-virus interaction and the biological sequelae of this encounter. Whereas the host may mount an immune response capable of viral neutralization, the induced immune response to the virus or to virus-induced neoantigens may

¹Abbreviations used in this paper: BSA, bovine serum albumin; ER, sheep erythrocyte rosette; HBcAg, hepatitis B virus-associated core antigen; HBsAg, hepatitis B virus-associated surface antigen; nER, rosettes formed between T lymphocytes and nSRBC; nSRBC, neuraminidase-treated SRBC; PBS, phosphate-buffered saline; PSG, Puck's saline G; RIF, Rosette Inhibitory Factor; SGOT, serum glutamic-oxaloacetic transaminase; SIg, surface membrane immunoglobulin; SRBC, sheep red blood cell.

play an integral role in the pathogenesis of the associated tissue injury (11, 12). These relationships and interactions are complicated by the reported suppressive effects of viruses on lymphocyte function (13, 14) as well as of viral infection on the immune response (15).

In the present study, we have focused our attention on the susceptibility of T-lymphocyte ER function to modulation during hepatitis B virus infection. We have discovered that the decrease in ER-positive lymphocytes characteristic of this disease is due to defective expression of ER function by circulating T lymphocytes rather than peripheral T lymphocytopenia. Two mechanisms responsible for suppression of T-lymphocyte ER function have been identified. A possible relationship between these mechanisms and the pathobiology of the associated disease has been suggested.

METHODS

Selection of patients. Young, adult, Caucasian men who were admitted to the hepatitis ward of the U. S. Naval Regional Medical Center, San Diego within 5 days of the onset of jaundice, and who were, at that time, serologically positive for the presence of HBsAg were requested to voluntarily enter the study. Informed consent was obtained, and 37 patients were studied. 10 were evaluated serially for a minimum of 12 wk. The remaining 27 were evaluated once or twice during the acute phase of viral hepatitis B. No medications other than occasional doses of aspirin and prochlorperazine were administered and these were withheld for 24 h before the lymphocyte studies.

Patient evaluation. In each case a diagnosis of acute viral hepatitis type B was made according to standard clinical criteria, including history and physical examination, complete blood count, urinalysis, and liver function tests consisting of determination of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase, direct and indirect bilirubin, and prothrombin time. Serum was obtained weekly for the quantitation of HBsAg by counterimmunoelectrophoresis (16) until negative results were obtained. Negative sera were confirmed by solid-phase radioimmunoassay (Austria, Abbott Laboratories, South Pasadena, Calif.). Antibody to HBsAg was determined by fluid-phase radioimmunoassay of double-antibody type (17). Percutaneous Menghini needle liver biopsies were obtained from the 10 patients followed serially at 3 and 12 wk after admission. The tissue was fixed in 10% formalin, and stained with hematoxylin and eosin as well as by reticulin and trichrome methods following routine processing.

Isolation of lymphocytes. Lymphocytes were isolated from peripheral blood collected into preservative-free heparin, as previously described (4). Briefly, the blood was mixed immediately in a syringe with 3.5 vol of carbonyl iron-dextran (Lymphocyte separating reagent, Technicon Instrument Corp., Tarrytown, N. Y.) and incubated at 37°C for 10 min, followed by 30 min at 22°C with slow inversion on a turntable mixer (10 rpm). The erythrocytes and phagocytic cells were sedimented first by placing the syringe horizontally for 15 min over a 3 × 3/4-inch ceramic magnet, then by placing the syringe vertically for 20 min through the center of the magnet. Sufficient supernate was expressed from the syringe into 1-ml plastic conical tubes (Fisher Scientific Co., Pittsburgh, Pa.) to provide 1.5–2 × 10⁶ leukocytes per tube. The cells were then sedimented and washed

four times with 1% bovine serum albumin in Puck's saline G (Grand Island Biological Co., Grand Island, N. Y.) (1% BSA-PSG) in a Fisher microcentrifuge for 60 s at 1,200 *g*. Lymphocyte recovery averaged 88% with an average purity of 93% of nucleated cells. Residual leukocytes were polymorphonuclear leukocytes. Viability was greater than 98%. Contamination of the mononuclear cell population by monocytes was evaluated by the peroxidase reaction according to Kaplow (18). In the eight subjects studied, $0.6 \pm 0.6\%$ of the mononuclear cells were peroxidase positive (range 0.0–1.8%) which suggests that 99.4% of the isolated mononuclear cells are lymphocytes.

Assay of SIg-positive lymphocytes. The lymphocytes were assayed for SIg in the cold (0–4°C) as previously described (4). To each of six tubes which contained $1.5\text{--}2 \times 10^6$ lymphocytes and approximately 2×10^7 carrier erythrocytes was added 50 μ l of a fluorescein-conjugated anti-immunoglobulin reagent of given specificity: IgG (γ -chain), IgA (α -chain), IgM (μ -chain), κ -light chain, λ -light chain, or Ig polyvalent, in 1% BSA-PSG, 0.03% sodium azide. After 30-min incubation of 0°C, the cells were washed two times in 1% BSA-PSG, once in 5% BSA-PSG, and the pellet was smeared on slides as for the preparation of blood smears.

The slides were dried overnight, fixed for 3 min in absolute methanol, stained for 5 min with acridine orange (0.35 μ g/ml, 0.1 M Tris-HCl, pH 8.0) to provide a faint counterstain, washed in distilled water and mounted in 1% Tris-HCl, pH 9.5, 90% glycerol. Slides were examined with a Zeiss RA microscope (Carl Zeiss, Inc., New York) equipped with HB-200 mercury arc illumination, no heat filter, a 490-nm interference type excitation filter, a NA 1.2–1.4 dark-field condenser, and a 530-nm barrier filter. SIg was scored from examination of 400 lymphocytes. Contaminating polymorphonuclear leukocytes were excluded cytologically by means of the faint acridine orange fluorescence of nuclei which is photo-inactivated within 3–5 s.

Fluorescein-conjugated reagents for SIg staining were prepared from specific antisera raised in goats or rabbits. Antisera to IgA and IgM were prepared by hyperimmunization of goats at monthly intervals with 100 μ g of a mixture of highly purified immunoglobulin of appropriate class in incomplete adjuvant. Antisera to IgM and to IgA were absorbed with IgG. Antiserum to IgG was prepared by immunization with the Fc fragment of IgG (19) and was absorbed with F(ab')₂. Each antiserum appeared specific by Ouchterlony and immunoelectrophoretic analyses and the lack of reactivity with purified immunoglobulins of the other classes coupled to Sepharose 4B beads (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) with cyanogen bromide (20). Anti-Ig polyvalent was prepared by chronic immunization of a single goat with 100 μ g each of IgG, IgA, IgM, IgD, κ -, and λ -light chains. The antiserum reacted with each of these classes of immunoglobulin and with light chains. Reactions with other serum proteins were not observed. Rabbit antiserum to λ -light chains and goat antiserum to κ -light chains (Meloy Laboratories Inc., Springfield, Va.) appeared specific for monoclonal immunoglobulins of appropriate light chain type by Ouchterlony analysis.

The gamma globulin fractions of the antisera were precipitated at 50% saturation with ammonium sulfate at 4°C and redissolved in 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3 (phosphate-buffered saline [PBS]). After three cycles of precipitation, the gamma globulin fraction was dialyzed against PBS and the protein (at 10 mg/ml) was conjugated with fluorescein isothiocyanate by dialysis (21) at

4°C for 20 h using 10 mg fluorescein isothiocyanate/ml protein solution. After dialysis against PBS, and absorption with 50 mg of guinea pig liver acetone powder/ml, the preparations were brought to 20% sucrose and clarified by ultracentrifugation at 68,000 *g* for 18–20 h. The monomeric 7S fraction was isolated by Sephadex G-200 (Pharmacia Fine Chemicals Inc.) chromatography and the FITC to protein molar ratio was determined spectrophotometrically to be approximately 5–6:1 (22). Each standard was utilized after repeated titration (23) to determine the concentration required to give a constant percentage of SIg⁺ cells. Each reagent gave a single plateau of constant percentage of SIg⁺ cells over a three- to fivefold range of concentration of reagent. The reagents were diluted in 1% BSA-PSG, 0.03% sodium azide. Purified antibody reagents were prepared from standard reagents by absorption to immunoadsorbents and elution with 3.5 M potassium iodide in 0.1% BSA, 0.01 M Tris-HCl, pH 8.0 (24), followed by gel filtration on Sephadex G-200 to recover the 7S fraction. The latter reagents were dialyzed, concentrated, and titered against normal peripheral blood lymphocytes as above. They gave results indistinguishable from the standard reagents. Specificity of each of the reactions was confirmed by neutralization of the reagents with specific soluble immunoglobulin or specific immunoglobulin coupled to Sepharose (25).

F(ab')₂ fragments of an independent anti-immunoglobulin antiserum reactive with all classes of immunoglobulin were prepared by pepsin cleavage according to Nisonoff et al. (26). This reagent was shown to be free from residual undigested IgG and contaminating Fc fragments by molecular exclusion chromatography on Sephadex G-150, Ouchterlony analysis, and immunoelectrophoresis, and was conjugated with fluorescein isothiocyanate as described above. The reagent was titrated as above and the plateau for SIg⁺ cells was employed in assays.

ER assay. Assay was that of Jondal et al. (1), performed as previously described (4). A lymphocyte-rich plasma depleted of phagocytic cells was produced as above with carbonyl iron from 2 ml of blood. The cells were sedimented for 10 min at 400 *g* and were then suspended in 15 ml of 0.75% ammonium chloride, 0.017 M Tris-HCl, pH 7.65, for 20 min at 37°C to lyse the erythrocytes. The lymphocytes were washed three times with RPMI-1640 (Grand Island Biological Co.). 5×10^6 lymphocytes (250 μ l) were mixed with 250 μ l of 0.5% washed sheep erythrocytes in PSG in a 15 ml conical tube, incubated for 5 min at 37°C, centrifuged for 4 min at 150 *g* and incubated for 18 h at 4°C. A drop of 0.02% Evans blue was added and the pellet was gently aspirated and transferred to a microslide. Viability was greater than 95% and purity was greater than 93% lymphocytes. 400 viable lymphocytes were counted and lymphocytes were scored positive if four or more erythrocytes were adherent and the lymphocytes exhibited definite surface scalloping. The arithmetic mean was calculated from triplicate assays and accepted if the SD was less than $\pm 5\%$.

Normal population value for SIg⁺ (B) and ER⁺ (T) lymphocytes. Analysis of peripheral blood lymphocytes from 30 normal healthy volunteers gave the values in Table I for SIg⁺ lymphocytes and ER-positive (ER⁺) lymphocytes. Within the context of this study, typical B cells were considered to be SIg⁺ER⁻. Typical T cells were considered as SIg⁻ER⁺. T-lymphocyte ER function was considered defective if the following conditions were met: (a) normal %SIg⁺ lymphocytes; (b) low %ER⁺ lymphocytes; (c) %SIg⁻ lymphocytes greater than %ER⁺ by at least 2 SD. The percent of lymphocytes defective in the expression of

ER function was calculated arithmetically: % defective = $[100 - (\%SIg^+ + \%ER^+)]$ %. The percent of cells that scored positive with the polyvalent immunoglobulin reagent was employed as %SIg⁺ in the above calculation since it was identical with the percent positive with the F(ab')₂ reagents in 15 randomly selected cases (see Results).

In vitro regeneration of ER function. Lymphocytes were analyzed for ER function and SIg after cultivation in vitro. Lymphocytes were washed and dispensed (1×10^6 lymphocytes/ml) into 12×75 -mm sterile polypropylene tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (50 µg/ml), l-glutamine (2 mM) and 20% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), or 20% autologous serum. After 24-h incubation at 37°C in 5% CO₂ in air, the lymphocytes were washed three times in RPMI-1640 at 250 g for 20 min and the cells were analyzed for SIg and ER markers.

Serum-mediated inhibition of ER function. Assay was performed as previously described (27). Briefly, lymphocytes were isolated from the peripheral blood of normal donors according to the method of Boyum (28) by centrifugation at 425 g onto a barrier of Ficoll (Sigma Chemical Co., St. Louis, Mo.) and Hypaque (Winthrop Laboratories, New York) adjusted to a density of 1.074 g/ml. The cells at the interface were washed twice in RPMI-1640, at 250 g and total leukocyte count, differential count, and viability (trypan blue exclusion) were determined. Absolute yield of lymphocytes was > 75% with 99–100% viability and > 90% purity.

Lymphocytes were suspended at 1×10^6 /ml in RPMI-1640 supplemented with penicillin, streptomycin, and l-glutamine as above and 2-ml aliquots were dispensed into 12×75 -mm sterile polypropylene culture tubes. Individual cultures received 0.5 ml of either normal autologous donor serum or test serum drawn at the time of the initial lymphocyte studies. After incubation for 24 h at 37°C in a humid atmosphere containing 5% CO₂ in air, pH 7.35 to 7.45, the cells were washed three times with RPMI-1640 at 250 g for 20 min and adjusted to a concentration of 1×10^6 cells/ml (viability > 95%, yield of cells > 85%). Experiments were performed in duplicate. Cells were assayed for SIg and ER markers.

The SD within individual assays was usually ± 2 –3% and always less than ± 5 %. The %ER⁺ lymphocytes after cultivation in test serum was compared with the %ER⁺ obtained after cultivation in control serum. The result was expressed as relative percent inhibition of ER function induced by test serum and was calculated as follows: % inhibition = $(C - X)/C \times 100$, where C equals %ER⁺ cells cultured in control serum (60–75%) and X equals %ER⁺ lymphocytes after culture in test serum. Significant inhibition was considered to be present if C differed from X by at least 2 SD.

Assay for nER⁺ lymphocytes. T lymphocytes were also identified by reference to their capacity to form rosettes (nER) with neuraminidase-treated SRBC (nSRBC) (29). 1 ml of 5% SRBC in RPMI-1640 was incubated with 75 µl of neuraminidase (500 U/ml) for 30 min at 37°C, washed with PSG and adjusted to a 0.5% concentration. To 250 µl of nSRBC was added 0.5×10^6 lymphocytes suspended in 250 µl of PSG. The mixture was incubated at 37°C for 10 min and centrifuged at 200 g for 5 min. The pellet was kept at room temperature for 60 min, at which time it was gently resuspended and the %nER⁺ lymphocytes were counted as for assay of ER⁺ cells.

Lymphocytotoxicity studies. All sera were tested for cytotoxic activity against the donor lymphocytes used in the

Rosette Inhibitory Factor (RIF) assays according to a modification of the microcytotoxicity method of Mittal et al. (30). Target lymphocytes were provided by the same individuals whose lymphocytes were used in the RIF assays. Incubation with test sera for 2 h at 4° and 25°C was followed by the addition of complement (rabbit serum that had been evaluated for use in lymphocytotoxicity assays [31] was generously supplied by Dr. Soldano Ferrone) and further incubation at room temperature for an additional 2 h. The percent of cytotoxicity was determined by microscopic determination of dye exclusion (eosin yellow). A minimum of 300 cells was counted for each condition and assays were performed in duplicate.

Antitissue antibody determinations. The presence of antitissue antibodies was evaluated (32). Sera were evaluated periodically for the presence of antinuclear, antismooth muscle, antimitochondrial, and antiparietal cell antibodies by indirect immunofluorescence utilizing 1:10 dilution of patient serum and immunochemically purified fluoresceinated antihuman IgG. Tissue substrates were acetone-fixed rat kidney (antinuclear), unfixed mouse kidney (antimitochondrial), and mouse stomach (antismooth muscle and antiparietal cell).

Quantitative immunoglobulin determinations. Sera were periodically examined by single-radial immunodiffusion for quantitative determination of IgG, IgA, and IgM (33). Assays were standardized with reference serum from Meloy Laboratories Inc.

Statistical analyses. Statistical significance of results was analyzed by two-tailed t test (34).

RESULTS

B- and T-lymphocyte distribution in normal controls. The relative distribution of peripheral blood B and T lymphocytes in 30 normal control subjects is summarized in Table I. Of the lymphocytes, 29.6±5.4% were designated B lymphocytes on the basis of SIg demonstrated with the anti-Ig polyvalent reagent. The accuracy of this observation was validated by agreement with three

TABLE I
Relative Distribution of Peripheral Blood B and T
Lymphocytes in 30 Normal Controls

	%*
Total B lymphocytes	
SIg ⁺ polyvalent	29.6±5.4
SIg ⁺ sum, κ- plus λ-light chains	27.4±5.8
SIg ⁺ sum, γ-, α-, μ-chains	28.5±5.7
B lymphocytes by class and type	
κ-chain type	14.2±3.3
λ-chain type	13.2±3.4
γ-chain class	14.5±4.7
μ-chain class	11.4±2.2
α-chain class	2.4±1.7
T lymphocytes	
SIg ⁻ polyvalent	70.4±5.4
ER ⁺	70.7±7.5

* Mean±SD.

independent parameters: (a) the sum of the percent lymphocytes positive with the anti- κ - and anti- λ - light chain reagents ($27.4 \pm 5.8\%$); (b) the sum of the percent of lymphocytes positive with the anti- γ -, anti- α -, and anti- μ -heavy chain reagents ($28.5 \pm 5.7\%$); (c) precise concordance between the %SIg⁻ lymphocytes ($70.4 \pm 5.4\%$) and the %ER⁺ ($70.7 \pm 7.5\%$). Specificity was further assured by assay of lymphocyte SIg with F(ab')₂ fragments of an independent anti-immunoglobulin polyvalent antiserum. Whereas $28.2 \pm 6.6\%$ were SIg⁺ with the standard anti-Ig polyvalent reagent in an additional 15 control subjects, $27.3 \pm 6.9\%$ were SIg⁺ with F(ab')₂ fragments as the fluorescein-conjugated reagent. The precise summation of light chain values, heavy chain values, and agreement of F(ab')₂ reagent effectively dismiss reactivity with Fc receptor.

T lymphocytes, identified as ER⁺, accounted for $70.7 \pm 7.5\%$ of the total lymphocyte population (Table I). The maximum range of variation in %ER⁺ lymphocytes within individual control subjects was less than $\pm 6\%$ when tested on multiple occasions. The sum of the %ER⁺ (T) lymphocytes plus the %SIg⁺ (B) lymphocytes determined by each of the four methods outlined above accounted for 98.1–100.3% of the total lymphocyte population. Monocyte contamination of the mononuclear cell preparations averaged 0.6% on the basis of peroxidase reactions. Significant distortion of the normal B/T lymphocyte distribution by the carbonyl iron-dextran isolation procedure is unlikely based on the 88.0% average lymphocyte recovery.

Defective T-lymphocyte ER function during acute viral hepatitis B. In marked contradistinction to the normal control group, each of the 37 patients with acute viral hepatitis B had a significant reduction in the relative number of ER⁺ (T) lymphocytes on one or more occasions during the course of their illness (Table II). In contrast, only minor variations in the relative num-

ber of SIg⁺ (B) lymphocytes were observed. Furthermore, the sum of %SIg⁺ plus %ER⁺ lymphocytes was frequently less than 100% indicative of the presence of lymphocytes deficient in both surface markers.

ER function was occasionally normal in patients in whom it was at other times depressed. Among 67 assays performed on the 37 patients, 55 displayed defective ER function while 12 were normal. Thus, although T-lymphocyte ER function was depressed in every patient at least once during the acute phase of illness, the abnormality was detectable in only 82.1% of the assays (55/67) and it affected an average of 24.5% of total lymphocytes (range 9.0–55.8%). This resulted in an average circulating concentration of 512 lymphocytes/mm³ of peripheral blood which appeared deficient for both markers based on an average total lymphocyte count of $2,089.2 \pm 526.4/\text{mm}^3$. These cells appeared to be generated at the expense of normal ER⁺ (T) lymphocytes since the relative ($47.8 \pm 13.2\%$) and absolute ($998.5 \pm 275.8/\text{mm}^3$) number of ER⁺ lymphocytes in the hepatitis group with defective lymphocytes were significantly lower than the relative (70.7 ± 7.5) and absolute ($1,395.9 \pm 426.7/\text{mm}^3$) number of ER⁺ lymphocytes in the controls. The number of SIg⁺ (B) lymphocytes in the two groups was virtually identical (Table II).

Intrinsic and extrinsic mechanisms responsible for the induction of defective T-lymphocyte ER function. When lymphocytes from 17 patients with defective T-lymphocyte ER function were washed free of serum constituents, incubated for 24 h in the presence of either fetal calf serum or autologous serum, and reexamined for the expression of SIg and ER, they were found to be separable into two distinct categories (Table III). Seven of these cases (group 1) displayed a significant ($P < 0.005$) increase in ER⁺ lymphocytes after cultivation in fetal calf serum, whereas no significant increase in ER⁺

TABLE II
Relative Distribution of Peripheral Blood B, T, and Defective Lymphocytes in Normal Individuals and 37 Patients with Acute Viral Hepatitis B

	Number	B lymphocytes (SIg ⁺)	T lymphocytes (ER ⁺)	Defective (SIg ⁻ , ER ⁻)*
		%‡	%	%
Total hepatitis assays	67	28.3 ± 7.5	51.2 ± 14.1 §	20.5 ± 13.6 §
Defective lymphocytes present	55	27.7 ± 7.5	47.8 ± 13.2 §	24.5 ± 12.8 §
Defective lymphocytes absent	12	30.7 ± 6.3	66.5 ± 5.9	None
Normal control assays	30	29.6 ± 5.4	70.7 ± 7.5	None
Age-, sex-matched control assays	10	30.5 ± 4.3	68.2 ± 4.2	None

* Calculated: % defective = $100 - (\% \text{Ig}^+ + \% \text{ER}^+)$.

‡ Mean ± 1 SD.

§ $P < 0.005$ when compared with control.

^{||} Absolute lymphocyte count: hepatitis = $2,089.2 \pm 526.4/\text{mm}^3$; controls $1,976.1 \pm 624.9/\text{mm}^3$.

lymphocytes occurred when cultured in autologous serum. Because of the regeneration of ER function of lymphocytes from group 1 in the absence of autologous serum the mechanism responsible for the initial defectiveness of ER function was tentatively considered to be extrinsic. In the 10 other cases (group 2) no significant increase in ER⁺ lymphocytes occurred after culture in either fetal calf or autologous serum. Hence the mechanism was tentatively regarded as intrinsic. The %ER⁺ lymphocytes from group 3 (hepatitis patients with normal ER function) or the control group, did not change significantly as a result of cultivation. The %SIg⁺ lymphocytes in all groups was comparable after cultivation in either fetal calf serum or autologous serum.

Evidence suggesting the existence of a serum inhibitor of T-lymphocyte ER function: RIF. The possibility that defective ER function of the extrinsic type was dependent upon autologous serum-induced attenuation of ER function was examined. Aliquots of normal lymphocytes were cultivated for 24 h, either in: (a) normal autologous control serum, or (b) test sera from patients in the above groups. After cultivation, expression of SIg and ER by the washed cells was assayed (Table IV).

The ER function of normal lymphocytes cultivated in the presence of serum from patients in group 1 with an extrinsic defect in ER function was reduced by greater than 2 SD in comparison with aliquots of the same lymphocytes cultured in control serum. In contrast, no reduction in the %ER⁺ lymphocytes was observed when the same cells were cultivated with sera from patients in group 2 with an intrinsic defect, group 3, or normal controls.

Direct evidence that lymphocytes with intrinsic and extrinsic defects in ER function possess T-lymphocyte surface markers. Lymphocytes from patients with both

TABLE III
Regeneration of ER Function In Vitro

Lymphocytes.....	Group 1	Group 2	Group 3	Control
Number of studies.....	7	10	5	5
Freshly isolated				
%SIg ⁺	31.1 ± 8.1	29.0 ± 5.1	34.2 ± 8.0	33.1 ± 3.7
%ER ⁺	34.9 ± 13.7	47.1 ± 7.9	62.6 ± 7.0	64.5 ± 7.2
% Defective	33.7 ± 14.6	23.4 ± 6.0	2.2 ± 0.9	2.4 ± 1.2
Incubated in fetal calf serum				
%SIg ⁺	21.5 ± 4.8	18.3 ± 6.3	19.3 ± 5.6	24.4 ± 5.3
%ER ⁺	62.7 ± 4.9	52.2 ± 14.1	64.3 ± 6.9	61.1 ± 6.5
% Regeneration ER*	+79.6 ‡	+10.8 §	+2.7 §	-5.3 §
Incubated in autologous serum				
%SIg ⁺	23.3 ± 3.5	17.3 ± 4.7	20.4 ± 2.8	21.6 ± 4.2
%ER ⁺	40.8 ± 5.8	48.1 ± 9.1	56.4 ± 9.5	59.4 ± 7.6
% Regeneration ER*	+16.9 §	+2.1 §	-9.9 §	-7.9 §

* % regenerated ER = $\frac{(\%ER \text{ incubated}) - (\%ER \text{ initial})}{\%ER \text{ incubated}} \times 100$.

‡ P < 0.005.

§ NS.

TABLE IV
Presence of RIF in the Serum of Patients with an Extrinsic Defect in T-Lymphocyte ER Function

Serum donor	Number	%ER ⁺	% Inhibition*	Inhibitory/ tested
Group 1	7	41.5 ± 9.1	38.4 ± 12.2 ‡	7/7
Group 2	10	60.9 ± 5.2	NS	0/10
Group 3	5	65.0 ± 3.8	NS	0/5
Controls	10	66.7 ± 4.5	NS	0/10
Lymphocyte donor		64.3 ± 5.2	—	—

* Normal peripheral blood lymphocytes incubated for 24 hr in test sera.

‡ P < 0.005.

extrinsic and intrinsic defects in ER function were assayed for the capacity to form rosettes (nER) with nSRBC (Table V). In both groups most SIg⁻ cells, including both ER⁺ and ER⁻ lymphocytes exhibited nER formation. These data provide direct evidence to further support the T-lymphocyte identity of the SIg⁻, ER⁻ lymphocytes observed in patients with acute viral hepatitis B.

Characterization of RIF. The temporal features of RIF-induced suppression of ER function was evaluated in vitro using six sera from RIF⁺ patients with an extrinsic defect in ER function. The relative inhibition of ER function of normal donor lymphocytes after incubation for 2-48 h was determined (Fig. 1). All RIF⁺ sera inhibited ER function with a characteristic latent period of approximately 4 h and maximum sustained effect within approximately 18 h. No inhibition was observed with sera from six RIF⁻ patients with an intrinsic defect in ER function.

To determine whether the biological effect of RIF⁺ sera was due to: (a) deficiency of a factor present in normal serum and required for normal ER function, or (b) the presence of a factor inhibitory to ER function, normal lymphocytes were cultured in the presence of RIF⁺ heterologous serum, normal autologous serum and

TABLE V
Rosette Formation with nSRBC by Defective Lymphocytes

Type of defect	Number	%SIg ⁺	Rosettes	Defective
			%	%
Extrinsic	5	28.4 ± 5.9	ER ⁺	35.8 ± 11.1
			nER ⁺	64.4 ± 4.7
Intrinsic	5	25.5 ± 4.8	ER ⁺	51.1 ± 4.7
			nER ⁺	72.5 ± 8.9
Control	7	28.2 ± 6.6	ER ⁺	70.8 ± 3.6
			nER ⁺	73.1 ± 4.5

TABLE VI
ER Function of Lymphocytes Cultured in RIF⁺ Serum with and without Supplementation by Normal Serum

Test serum donor number	%RIF ⁺ serum in culture	%Autologous (RIF ⁻) serum in culture	%ER ⁺	%Relative inhibition
8	20	None	54.5±3.4	28.0
8	10	None	56.0±2.1	25.2
8	10	10	50.3±2.3	33.3
—	None	10	74.8±1.5	—
—	None	20	74.9±2.1	—
10	20	None	50.0±2.0	33.4
10	10	None	49.8±1.8	37.7
10	10	10	50.3±2.4	33.3
—	None	10	74.8±1.5	—
—	None	20	74.9±2.1	—
19	20	None	57.5±2.5	23.2
19	10	None	57.0±2.6	23.8
19	10	10	59.0±3.7	21.3
—	None	10	74.8±1.5	—
—	None	20	74.9±2.1	—

mixtures of the two. As shown in Table VI, RIF⁺ serum consistently induced a decrease of ER⁺ cells which was not modified by the simultaneous presence of normal autologous serum.

Relationship between RIF and HBsAg. 1 ml each of three HBsAg-positive sera, also positive for RIF activity, was centrifuged at 114,000 *g* at 4°C for 90 min to pellet the HBsAg. The sera were recovered in two 0.5-ml fractions and were assayed for HBsAg and for RIF activity (Table VII). 80% of the HBsAg was recovered in the pellet, whereas RIF activity was recovered in the supernatant fractions. RIF appeared to be concentrated in the supernate as indicated by the increased RIF activity as compared to the same volume of the initial serum. In separate studies, RIF was found to possess the buoyant density of low density lipoprotein

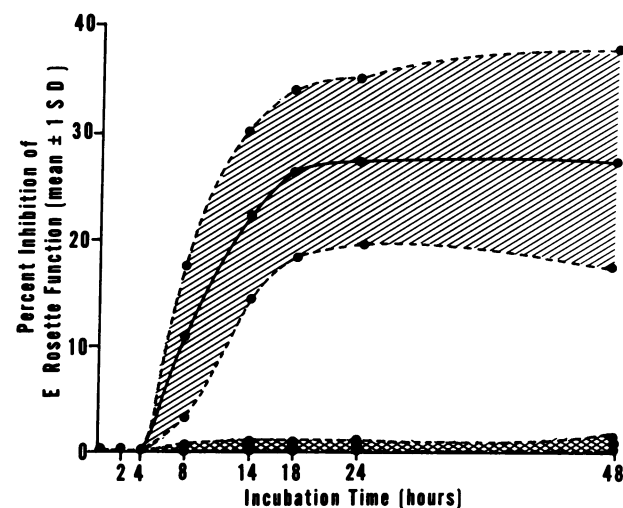


FIGURE 1 Effect of group 1 and group 2 sera on ER function of normal T lymphocytes. Sera from six group 1 patients with an extrinsic lymphocyte defect were incubated with normal lymphocytes for the times indicated. The lymphocytes were washed and ER function was assessed. Sera from six group 2 patients possessing an intrinsic lymphocyte defect were also assayed. Results were compared with the ER function of lymphocytes cultured in normal autologous serum. Shaded area, group 1 sera; crosshatched area, group 2 sera.

(1.050±0.004 *g/cm*³) and to be devoid of nonlipoprotein constituents by biochemical and immunochemical techniques (27).

Properties of RIF activity. Fresh serum samples in which RIF activity was detectable were repeatedly frozen and thawed without loss of RIF activity. Three RIF⁺ sera were heated at 56°C for 45 min without loss of activity. No loss of RIF activity was observed after dialysis of three additional RIF⁺ sera against PBS, pH 7.4.

Lymphocytotoxic activity, immunoglobulin concentration, and antitissue antibodies in sera with and without RIF activity. None of the sera in this study were di-

TABLE VII
Disposition of RIF Activity after Ultracentrifugation of Serum

Serum donor number	RIF activity					
	Whole serum		Supernatant fraction		Pellet fraction	
	%ER ⁺	Inhibition	%ER ⁺	Inhibition	%ER ⁺	Inhibition
		%		%		%
10	54.1±2.6	14.6	35.2±2.5	44.4	64.2±3.6	0
14	50.2±3.1	20.7	35.1±1.6	44.5	57.8±2.1	8.7
19	53.2±1.4	14.4	38.4±2.6	39.3	58.1±3.5	8.2
Autologous	63.3±1.7	—	ND	—	ND	—

ND, not done.

TABLE VIII
Classification of Patients with Viral Hepatitis B According to Presence and Type of Defective Lymphocytes and the Presence of Serum RIF

Group	Number	B Lymphocytes %SIg ⁺	T Lymphocytes %ER ⁺	Defective %SIg ⁻ , ER ⁻	Regeneration of ER	Serum RIF activity
1	23	29.2±7.8	45.1±13.5	25.7±13.7	7/7	23/23
2	32	26.4±7.1	49.8±12.6	23.6±12.0	0/10	0/32
3	12	30.7±6.3	66.5±5.9	None	0/5	0/12
Control	10	30.5±4.3	68.2±4.2	None	0/5	0/10

rectly cytotoxic for the standard normal lymphocytes used in the RIF assay as determined by trypan blue exclusion. Complement-dependent lymphocytotoxicity assays were performed to assess the presence of anti-lymphocyte antibodies which might have been responsible for the RIF effect. None of the RIF⁺ sera were cytotoxic for any of the standard normal lymphocytes used in the RIF assays.

Serum levels of IgG, IgA, and IgM were determined weekly for the first month of the study and subsequently at the time of the B- and T-lymphocyte evaluation. No significant or consistent changes were noted.

Serum samples obtained at the time of each lymphocyte study were also examined for the presence of anti-nuclear, antimitochondrial, antismooth muscle, and anti-parietal cell antibodies. All samples were negative at a dilution of 1:10.

Classification of patients according to type of defect in ER function. 67 serum samples obtained from 37

patients at the time of lymphocyte assay were analyzed for RIF activity. RIF was present in 23 of the 55 cases with defective ER function (41.8%) and absent in the remaining patient groups and controls. The patients could be resolved into three categories on the basis of the presence of defective ER function and serum RIF activity (Table VIII).

The lymphocytes from all patients in group 1 had defective ER function and serum RIF activity was simultaneously present. Lymphocytes from seven of these patients were assayed for ER function after cultivation in the absence of autologous serum (see also Table IV) and all seven displayed the ability to regenerate ER function under these conditions.

The lymphocytes from all patients in group 2 had defective ER function but serum RIF activity was absent. Regeneration of ER function was not demonstrable in any of the 10 cases from this group in which it was assayed.

TABLE IX
Variations in the Pattern of Defective Lymphocytes during the Course of Acute Viral Hepatitis B

Patient	Weeks*	Type of lymphocyte defect	SGOT	Histo-logical diagnosis‡	HBsAg	HBsAb	Patient	Weeks*	Type of lymphocyte defect	SGOT	Histo-logical diagnosis‡	HBsAg	HBsAb
			IU§							IU§			
1	2	None	475	AVH	+	-	6	2	Intrinsic	200	AVH	+	-
	4	ND	82	-	-	-		4	Extrinsic	87	-	+	-
	12	Intrinsic	35	NL	-	+		12	Intrinsic	29	NL	-	+
2	2	Extrinsic	588	AVH	+	-	7	2	Extrinsic	410	AVH	+	-
	4	Extrinsic	120	-	+	-		4	Intrinsic	612	-	-	-
	12	Extrinsic	624	UH	-	+		12	Intrinsic	37	NL	-	-
3	2	Extrinsic	590	AVH	+	-	8	2	Extrinsic	483	AVH	+	-
	4	Extrinsic	87	-	+	-		4	Intrinsic	390	-	-	+
	12	Extrinsic	239	UH	-	-		12	Intrinsic	20	NL	-	+
4	2	None	800	AVH	+	-	9	2	None	>1,000	AVH	+	-
	4	None	>1,000	-	+	-		4	Intrinsic	86	-	+	-
	12	Intrinsic	32	RH	-	-		12	Intrinsic	24	RH	-	-
5	2	Extrinsic	320	AVH	+	-	10	2	Extrinsic	>1,000	AVH	+	-
	4	Extrinsic	38	-	+	-		4	None	206	-	+	-
	12	Intrinsic	26	NL	-	-		12	None	31	NL	-	-

* Weeks after onset of jaundice.

‡ AVH, acute viral hepatitis; NL, normal; RH, resolving acute hepatitis; UH, unresolved hepatitis.

§ Upper limits of normal = 40 IU/ml.

Group 3 cases as well as the controls had normal ER function and no serum RIF activity. No patients or controls were observed to have serum RIF activity in the absence of defective ER function. There was no correlation between the presence of defective ER function or RIF activity and the presence of HBsAg in the serum.

These data suggest that the coexistence of serum RIF activity and deficient ER function is causal and suggest that an extrinsic mechanism is responsible for defective T-lymphocyte function in these patients. In the absence of serum RIF activity other mechanisms must be implicated and these may be intrinsic to the lymphocyte.

Association of intrinsic and extrinsic defects in ER function with the course of the acute viral hepatitis B. Among 10 patients followed serially through the course of acute viral hepatitis B and recovery (Table IX), 2 patients (2 and 3) had elevated SGOT levels 12 wk after the onset of jaundice. These same patients had histopathological evidence of unresolved hepatitis, characterized by portal lymphoid infiltrate with extension into lobular parenchyma, mild piecemeal necrosis of the limiting plate, and focal hepatocytolysis. The remaining eight patients had normal SGOT levels at 12 wk. The liver biopsies of six of these eight patients were entirely normal at 12 wk while the other two (4 and 9) revealed slight persistent portal and parenchymal lymphoid infiltrate without hepatocytolysis or piecemeal necrosis consistent with the resolving phase of acute viral hepatitis.

During the first 4 wk of illness, the subsequent course of disease could not be distinguished on the basis of the type of defective T lymphocytes present. At 12 wk, however, a clear-cut distinction could be made between those patients with resolved or resolving hepatitis, none of whom had an extrinsic defect, and those with unresolved hepatitis, all of whom had an extrinsic defect (Table X). Only patients with unresolved hepatitis had an extrinsic defect in T-lymphocyte ER function at every sampling interval, in contrast to random variation in type of defect in the cases that resolved. Despite existing or imminent clinical recovery, an intrinsic defect in ER function persisted in seven of the eight patients with resolved or resolving hepatitis.

DISCUSSION

During the acute phase of hepatitis B virus infection, reduced numbers of SIg⁻ peripheral blood lymphocytes exhibit ER formation, whereas the number of SIg⁺ (B) lymphocytes remains relatively normal. This results in significant numbers of lymphocytes deficient for both markers. However, virtually all SIg⁻ cells rosette with neuraminidase-treated erythrocytes, suggesting that the functionally deficient cells are T lymphocytes, and that the relative and absolute number of circulating T lymphocytes remains normal in this disease in contrast to a previous report (8).

TABLE X
Defective ER Function and Clinical Resolution of Disease

Type of lymphocyte defect	Resolved or resolving hepatitis			Unresolved hepatitis		
	2 wk	4 wk	12 wk	2 wk	4 wk	12 wk
Extrinsic	4	2	0	2	2	2
Intrinsic	1	3	7	0	0	0
None	3	2	1	0	0	0

phocytes remains normal in this disease in contrast to a previous report (8).

Two distinct mechanisms are implicated in the generation of ER⁻ T lymphocytes. One is causally related to an extrinsic serum factor, whereas the other mechanism appears to be intrinsic to the defective lymphocyte. The extrinsic mechanism appears to operate via an interaction between T lymphocytes and a humoral substance referred to as RIF. Substantiation of this hypothesis is provided by two lines of evidence: (a) lymphocytes with the extrinsic defect reacquire ER function when cultured in the absence of autologous serum; and (b) such sera readily suppress the ER function of T lymphocytes from normal donors. In contrast, lymphocytes with an intrinsic defect do not regenerate ER function *in vitro*, nor does the autochthonous serum inhibit ER function of normal lymphocytes.

Both mechanisms appear to induce functionally deficient T lymphocytes rather than functionally defective (SIg⁻) B lymphocytes or some alternative cell type. This is suggested by reference to the observed deficiency of ER⁺ lymphocytes in the face of normal numbers of SIg⁺ lymphocytes. Further support is provided by the observation that both types of defective lymphocytes are capable of forming rosettes with nSRBC, a reasonably T-lymphocyte-specific phenomenon (9). The possibility that such defective lymphocytes might represent monocytes is rendered unlikely by the demonstrated effectiveness of the iron-dextran lymphocytes isolation method to reduce peroxidase-positive cells (18) to less than 1%.

The existence of multiple mechanisms for modification of T-lymphocyte ER function is consistent with the observations that this function depends upon critical metabolic processes including oxidative phosphorylation (35), glycolysis (1), microfilament function (36), protein and nucleic acid synthesis (3), divalent cation concentration (36), and cyclic nucleotide levels (4). Modification of one or more of these variables could be responsible for the observed attenuation of normal ER function.

Many of the metabolic processes that influence ER function also influence other well-established T-lympho-

cyte functions such as mitogen-induced blastogenesis (37–39), lymphocyte-mediated cytotoxicity (40), the plaque-forming cell response to thymus-dependent antigens (41, 42), and lymphokine-induced inhibition of macrophage migration (43). ER formation by a T-cell line is associated with release of an immunosuppressive factor (44). The possibility that certain forms of diminished ER function may reflect significant functional properties remains to be established but is suggested by observations that lymphocytes from patients with viral hepatitis B also are hyporesponsive to the T-cell mitogen phytohemagglutinin (45) although they may be cytotoxic to autochthonous liver cells (46). Diminished ER function has been described in a variety of diseases which may have associated immunological aberrations: these include cancer (6), autoimmune diseases (7), immunodeficiency states (5, 47), marijuana smoking (48), inflammatory bowel disease (49), alcoholic liver disease (50), and other viral infections (6).

It is now evident that defective (ER⁻) T lymphocytes occur at one time or another in every patient studied, and they have persisted for at least 12 wk after the onset of jaundice in 9 of 10 subjects followed serially. A distinction was observed between two patients with unresolved hepatitis and eight others whose illness was resolved or resolving. The T lymphocytes from patients with unresolved hepatitis displayed an extrinsic defect at every sampling interval. In contrast, none of the 8 patients with transient disease had an extrinsic T-lymphocyte defect, or RIF at 12 wk. Further long-term clinical studies will be required in order to establish the consistent association of the extrinsic mechanism and RIF with the development and course of chronic hepatocellular injury after hepatitis B virus infection.

The existence of a new serum factor, RIF, evolved during this study. This represents a substance(s) in the serum of patients with an extrinsic T-lymphocyte defect which suppresses T-lymphocyte ER function *in vitro*, and presumably *in vivo* as well. Normal serum does not correct the effect of RIF, which suggests that this effect does not reflect absence of a normal factor required for ER expression, but rather the presence of a suppressive factor. That RIF exerts a metabolic effect, rather than simple steric hindrance is suggested by the 4-h latent period and the time-dependent attenuation of ER function during cultivation. A similar delayed inhibition of ER function is seen when lymphocytes are incubated *in vitro* with cholera enterotoxin, an agent known to induce a delayed rise in intracellular adenylyl cyclase and cyclic AMP levels (4). The partial rather than complete inhibition of ER function may reflect the concentration of RIF, subsets of T lymphocytes differing in ER function or susceptibility to RIF. Heterogeneity of ER function is indicated inasmuch as only 25–30% of peripheral

blood lymphocytes form ER immediately after the cells are mixed, whereas 4–18 h of incubation may be required for ER formation by the entire T-lymphocyte population.

RIF is heat stable, freeze-thaw stable, nondialyzeable, and is not a lymphocytotoxic antibody. Furthermore, it does not appear to represent intact HBsAg particles since there is no correlation between the presence of HBsAg and RIF; also RIF activity and HBsAg can be separated by ultracentrifugation. When HBsAg-positive serum is subjected to velocity sedimentation in the ultracentrifuge, antigenic activity may remain at the top of the gradient (17), presumably representing degradation products. In view of the observation that dialyzeable and nondialyzeable fractions of autoclaved measles virus can inhibit phytohemagglutinin-induced lymphocyte proliferation (13), we have considered whether RIF could be due to analogous HBsAg degradation products or constituent polypeptides. The presence of serum proteins on the surface of purified HBsAg particles has been reported (51) and two appear to be apolipoproteins. In separate studies we have demonstrated that RIF is a discrete and homogeneous lipoprotein with the density (1.050 ± 0.004 g/cm³), size (approx. 4×10^6 daltons), and immunochemical characteristics of a low density lipoprotein and appears to possess apolipoprotein chains AII, B, and CIII (27). Whether RIF represents viral-coded lipoprotein, molecular debris resulting from hepatocellular injury, or an independent entity must be established.

The occurrence of immunoregulatory factors in human serum has been suggested by a number of investigators (52–58). RIF does not appear to be identical to previously described immunoregulatory factors. It is nondialyzeable, which suggests that it is neither an immunoregulatory α -globulin peptide (57, 58) nor an adrenocortical steroid (55). RIF⁺ sera are not directly cytotoxic for the donor lymphocytes used in the RIF assay, nor do they mediate complement-dependent cytotoxicity against these lymphocytes, which suggests that lymphocytotoxic antibodies (52, 53) are not responsible for RIF effect. The physicochemical properties of RIF (27) clearly distinguish it from immunoglobulins, immunoregulatory α -globulin (56), and other serum glycoproteins (54), but rather establish RIF as a bioregulatory lipoprotein.

The present study indicates that infection with the hepatitis B virus is associated with two distinctive types of defective T-lymphocyte function. The mechanisms responsible for the induction of these defects appear to be quite independent, and it is suggested that one type of defect attributable to a serum factor may be associated with protracted disease. Further elucidation of these mechanisms may eventually provide a cogent basis for understanding the immunobiology of the host, his re-

sponse to the challenge of viral infection, and the pathogenetic basis for diseases associated with potentially persistent noncytopathic viral agents (59).

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