LYMPHOCYTE E ROSETTE INHIBITORY FACTOR:
A REGULATORY SERUM LIPOPROTEIN*

BY FRANCIS V. CHISARI AND THOMAS S. EDGINGTON

(From the Department of Molecular Immunology, Scripps Clinic and Research Foundation,
La Jolla, California 92037)

The formation of nonimmune rosettes with sheep erythrocytes (E)\(^1\) is a
dynamic, energy dependent function (1) of human T lymphocytes. It is subject to
modulation by intracellular cyclic nucleotide levels (2, 3) and requires intact
glycolytic (4), protein, and nucleic acid (5) synthetic pathways for full expres-
sion. It is therefore reasonable to suspect that E rosette function may be
susceptible to inhibition by in vivo events which influence these metabolic
pathways. Defective T lymphocyte E rosette function is observed in association
with several diseases including cancer (6), autoimmune diseases (7), and viral
infections (6); however, the specific mechanisms responsible for the observed
defects of E rosette function in these diseases have not been established.

The hepatitis B virus may lead to either transient or persistent infection, and
the associated diseases appear to be mediated by the host response (8). During
hepatitis B virus infection a subpopulation of T lymphocytes, functionally
deficient in respect to E rosette function and lacking easily identifiable surface
membrane immunoglobulin, has been consistently observed.\(^2\)\(^3\) Two independ-
ent mechanisms appear to be associated with the generation of functionally
defective T lymphocytes during viral hepatitis B. One mechanism, associated
with persistent hepatitis, appears to be mediated by serum factor(s) extrinsic to
the lymphocyte, and such cells have been referred to as extrinsic null cells.\(^3\)
These cells reacquire normal E rosette function when cultured in normal serum.
Cultivation of normal lymphocytes with serum from patients with extrinsic null
cells induces a similar functional defect in E rosette function (9). The factor
responsible for induction of defective E rosette function we have designated

---

* Supported by U. S. Public Health Service research grant CA-14346 and contract AI-32509.
Publication EP-995.

1 Abbreviations used in this paper: E, sheep erythrocytes; HBcAg, hepatitis B core antigen;
HBsAg, hepatitis B surface antigen; LDL, low-density lipoprotein; PBS, phosphate-buffered
saline; RIF, rosette inhibitory factor; VLDL, very low-density lipoprotein.

2 Chisari, F. V., J. S. Routenberg, and T. S. Edgington. 1975. Human T lymphocyte “E” rosette
function. II. Null cell generation in association with Hepatitis B virus infection. Submitted for
publication.

3 Chisari, F. V., J. S. Routenberg, and T. S. Edgington. 1975. Human T lymphocyte “E” rosette
function. III. Two mechanisms of null lymphocyte generation associated with viral hepatitis B.
Submitted for publication.
rosette inhibitory factor, RIF. It is nondialyzable, stable upon heating to 56°C for 30 min, not a complement dependent lymphocytotoxic antibody, physically separable from HBsAg, and is of low density.

In the present study RIF has been isolated and characterized, and the effect of this factor on certain lymphocyte functions is described. RIF appears to represent a unique species of low-density lipoprotein (LDL) which binds to lymphocytes and induces attenuated E rosette function secondary to modulation of cellular events. RIF appears to selectively suppress certain T lymphocyte functions including E rosette function and to a lesser degree the capacity to respond in mixed lymphocyte reactions.

Materials and Methods

Sera. Blood was collected from young, otherwise healthy adult men with acute viral hepatitis B and from age and sex matched controls previously described.

Serum was promptly removed by centrifugation and stored at −20°C. RIF+ and RIF− sera were used individually, and aliquots were combined to form independent pools.

Lymphocytes. Lymphocytes were isolated from the peripheral blood of normal donors according to the method of Boyum (10) by centrifugation at 20°C and 425 g onto a barrier of nine parts 8 g/100 ml Ficoll (Sigma Chemical Co., St. Louis, Mo.) and two parts 50% Hypaque (Winthrop Labs., New York, NY) adjusted to a density of 1.074 g/ml (refractive index 1.3470) with 8% Ficoll. The cells at the interface were washed twice in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) at 250 g for 10 min, 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.

RIF Assay. Lymphocytes were suspended at 1.5 × 10⁶/ml in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (50 µg/ml), and L-glutamine (2 mM) (Flow Laboratories, Inc., Rockville, Md.). Cells were dispensed in 200 µl aliquots into sterile tissue culture plates with flat bottom wells (Microtest II, Falcon Plastics, Oxnard, CA) containing 50 µl of whole serum or fractions. When fractions were assayed, complete culture media supplemented with 20% heat inactivated fetal calf serum (Flow Laboratories, Inc.) was used. When whole human serum was assayed the sample served as the protein source. Culture plates were covered with loose fitting sterile lids and incubated for 18 h at 37°C in a humid atmosphere containing 5% CO₂ in air.

For assay of percent E rosette positive lymphocyte, cells were gently suspended, transferred to V-bottom microtiter plates (Cooke Engineering, Alexandria, Va.), and washed three times with 200 µl RPMI-1640 by centrifugation at 200 g for 3 min. After the last wash all but 50 µl of RPMI-1640 was removed, and 50 µl of a thrice washed 1.25% suspension of fresh E in RPMI-1640 was added. The plate was sealed, incubated for 10 min at 37°C, centrifuged for 2 min at 200 g, and incubated 18 h at 4°C. 5 µl of 0.02% trypan blue was added, and the pellet was gently aspirated and transferred to a microscope slide. Viability was always greater than 95%. 400 viable lymphocytes were counted and scored as previously described (2). The arithmetic mean was calculated from duplicate assays and accepted if the SD was less than ±5%.

Results were expressed as relative percent inhibition of E rosette positive cells induced by test serum or serum fraction as compared to control and were calculated:

\[
\text{inhibition} (\%) = \frac{C - X}{C} \times 100,
\]

where C equals percent E rosette positive lymphocytes cultured in control sample (60–75%) and X equals percent E rosette positive lymphocytes following culture in test sample. Unless otherwise stated both phosphate-buffered saline (PBS) and autologous serum served as controls in all assays, and percent rosette positive cells in both control assays agreed within ±2.0%. Experiments were performed in duplicate, and significant inhibition was considered to be present if C differed from X by at least 2 SD.
**Single Analytical Flotation and Isopycnic Banding.** 1.5 ml of serum was adjusted to a density of 1.15 g/cm³ with KBr, placed in a 5-ml ultracentrifuge tube, and overlaid with 1.5 ml of 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3 PBS adjusted to a density of 1.10 with KBr. The tube was then filled with 2.0 ml of PBS and centrifuged in a Spinco SW-50.1 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.) at 189,000 g average for 18 h at 20°C. Six 0.8-ml fractions were collected sequentially from the tops of tubes and dialyzed against three changes of 100 vol of PBS for 24 h. Aliquots were analyzed for RIF activity, lipoproteins, serum proteins, and hepatitis B virus surface antigen (HBsAg).

RIF⁺ fractions were pooled, adjusted to a density of 1.063 g/cm³ with KBr, and subjected to isopycnic density gradient ultracentrifugation in a Spinco SW-50.1 rotor at 114,000 g average for 72 h and 20°C. Serial fractions of 0.2 ml were collected from the top, and absorbance was monitored at 280 nm. The density of fractions was calculated from refractive index. Fractions were dialyzed and characterized as above.

**Lipoprotein Isolation by Sequential Flotation.** Conventional lipoprotein classes of defined density were isolated from pools of RIF⁺ and RIF⁻ sera by sequential flotation according to the method of Hatch and Lees (11).

**Analytical Sequential Isopycnic Ultracentrifugation.** The density of RIF was estimated by sequential isopycnic density gradient ultracentrifugation of RIF⁺ very-low density lipoprotein (VLDL) plus LDL derived by sequential flotation. Samples were adjusted to 1.050 g/cm³ with KBr and centrifuged in a SW-50.1 rotor at 217,000 g average for 74 h at 18°C. Fractions were collected as above, and absorbance was monitored at 280 nm, refractive index, and RIF activity was measured.

**Preparative Isopycnic Density Gradient Ultracentrifugation.** 200 ml of serum was adjusted to a density of 1.10 g/cm³ with KBr, and 5-ml aliquots were layered under 7.0 ml of PBS adjusted to 1.050 g/cm³ with KBr and centrifuged in a SW-41 rotor at 149,800 g average for 65 h at 18°C. Fractions (0.4 ml) were collected, and absorbance was monitored at 280 nm and refractive index. Fractions were pooled, and again centrifuged as above. Those fractions with a density between 1.040 and 1.060 g/cm³ were pooled and dialyzed.

**Ion Exchange Chromatography.** LDL of 1.050±0.004 g/cm³ from RIF⁺ and RIF⁻ sera were eluted with 0.005 M Tris-HCl, pH 8.3, and applied to either a 2.5 x 30-cm preparative column or a 0.9 x 10-cm analytical column of DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, Cal.) at 20°C and equilibrated with the same buffer. Proteins were eluted in a stepwise fashion with increasing concentrations of KBr in 0.005 M Tris-buffer, and the effluent was monitored at 280 nm. Fractions were concentrated to the initial sample volume, dialyzed, and assayed for RIF activity.

**Molecular Exclusion Chromatography.** Samples were applied to either a 2.5 x 60-cm preparative column or a 0.9 x 10-cm analytical column of Bio-Gel A-5.0, 200-400 mesh (Bio-Rad Laboratories) at 20°C and equilibrated with PBS. The effluent was monitored at 280 nm, and fractions were collected, concentrated, and assayed for RIF activity.

**Imunochemical Characterization of RIF.** Association of RIF activity with specific molecules was analyzed in double antibody immunoprecipitation assays. Heat inactivated antisera specific for human serum proteins including rabbit and goat antisera to albumin, IgG, IgA, IgM, polyvalent immunoglobulin, transferrin, alpha-1 antitrypsin, haptoglobin, fibrinogen, and beta lipoprotein (Meloy Laboratories Inc., Springfield, Va.), as well as with antisera to hepatitis B surface (HBsAg) and core (HBcAg) antigens, were employed. Aliquots (100 µl) of a pool of RIF⁺ serum, diluted to approximately 80% of maximum RIF activity (1:3), was added to 20 µl of each antisera and incubated 1 h at 37°C and 18 h at 4°C. 20 µl of heat-inactivated precipitating antiserum, either goat antirabbit gamma globulin or rabbit antigoat gamma globulin, was added, and incubation was continued for an additional 24 h at 4°C. After centrifugation at 7,000 g for 10 min (Microfuge, Fisher Scientific Co., Pittsburgh, Pa.), the supernates were assayed for residual (free) RIF activity, and the percent RIF bound was calculated.

Similar binding studies were performed using purified RIF diluted to approximately 80% of activity (20 ng/ml). Antisera to the apolipoproteins A₁, A₂, B, C₃, C₄, and D (kindly provided by Dr. P. Alaupovic, Oklahoma Medical Research Foundation, Oklahoma City, Okla.) were also employed. These antisera have been shown to be specific for the specific apolipoproteins by Ouchterlony and immunoelectrophoretic analyses (12).
Additional characterization of RIF and its equivalent biologically inactive lipoprotein fraction from normal serum was achieved by double diffusion in 1% agarose gels using antisera to alpha lipoprotein and beta lipoprotein (Meloy Laboratories Inc., Springfield, Va.).

Antisera to RIF and the equivalent RIF negative material were raised in adult male New Zealand white rabbits according to the following schedule: an initial injection of 1 μg of immunogen in complete Freund's adjuvant was made into the popliteal lymph nodes. This was followed at 2-wk intervals by multiple intracutaneous paraspinal injections of the immunogen in complete Freund's adjuvant. The antisera obtained were compared in double diffusion experiments with antisera to alpha and beta lipoproteins, as well as with antisera to specific apolipoproteins provided by Dr. P. Alaupovic.

**RIF Effect on E Rosette Function.** The dose response of RIF on E rosette function of lymphocytes was determined as for the RIF assay using RIF* and RIF' sera and purified RIF diluted in PBS, fetal calf serum, and in RIF' human serum. Kinetics of inhibition of E rosette function was determined after incubation with normal lymphocytes for varying periods at 37°C. The lymphocytes were washed and assayed for E rosette function as described above.

Recovery from RIF effect was assayed after addition of 50 μl purified RIF at 20 ng protein/ml to lymphocytes (4 ng RIF protein/ml culture) and incubated for 24 h. The lymphocytes were washed three times in complete media and returned to culture in the absence of RIF, and E rosette function was assessed at intervals thereafter.

**Binding of RIF by Lymphocytes.** 100-μl aliquots of purified RIF at 20 ng protein/ml in complete medium was added to varying numbers of lymphocytes in microcentrifuge tubes. The cells were suspended and incubated for 1 h at 37°C and then removed by centrifugation at 7,000 g. The percent RIF activity remaining in the supernates was determined from standard dilutions of purified RIF, and percent bound was calculated. The maximum number of RIF molecules capable of being bound per lymphocyte was determined by least square matched linear regression analysis of RIF bound per lymphocyte vs. the bound/free ratio of RIF. The estimated mean binding constant was calculated from k = [RIF-R]/[RIF][R], where [R] represents the molar concentration of lymphocyte receptors for RIF.

**Mixed Lymphocyte Reactions.** Two-way, mixed lymphocyte cultures were performed by a modification of the microculture method of Thurman et al. (13). Peripheral blood lymphocytes were isolated from two standard unrelated donors by the Ficoll-Hypaque method as described above. Cells were adjusted to 1.0 × 10⁶/ml in complete media; and equal volumes from each donor were mixed. Aliquots (200 μl) were dispersed into microculture plates containing 20 μl of either RIF* serum, RIF' serum, fetal calf serum, purified RIF, or the equivalent material isolated from RIF' serum. The cultures were incubated for 96 h at 37°C in a humidified atmosphere containing 5% CO₂ in air at the end of which 1 μCi of [³H]methylthymidine (sp act 2 Ci/mmol) was added. After 18 h incubation the cultures were harvested into glass fiber filters utilizing a multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, Md.). The filters were dried, dispensed into vials containing 3 ml of scintillation cocktail composed of Omnifluor (New England Nuclear, Boston, Mass.) in toluene, and counted. Experiments were performed in triplicate.

**Mitogen Responsiveness.** The DNA synthetic response of lymphocytes to stimulation by phytohemagglutinin (PHA) was assayed by the microculture method of Hartzman et al. (14) using a system similar to that described above for the mixed lymphocyte reaction. Peripheral blood lymphocytes were suspended in complete culture media at a concentration of 0.5 × 10⁶/ml. Aliquots of 200 μl were dispensed into microculture plates which contained 20 μl of RIF* sera, fractions or control materials, and 1 μl (0.6 μg) of PHA-P (Difco Laboratories, Detroit, Mich.), an optimal concentration, was added. After 48 h, 4 μCi of [³H]methylthymidine (50 mCi/mmol) was added, and incubation was continued for an additional 18 h. The cells were harvested and counted as described above. Assays were performed in triplicate.

**Lipoprotein Electrophoresis.** Lipoprotein electrophoresis was performed on cellulose acetate in 0.025 M barbital buffer, pH 8.8, on a Beckman Model R-101 Microzone apparatus. After electrophoresis at 12.8 V/cm for 45 min, the strips were dried and stained with fat red.

**Immunoelectrophoresis.** Microimmunoelectrophoresis was performed in 1% agarose on 1 x 3 inch microslides at 6 V/cm for 80 min. Precipitin patterns were developed with 100 μl of antiwhole human serum (Meloy Laboratories) by incubation for 24 h at room temperature.

**HBsAg.** HBsAg was assayed by the solid phase assay (Austria, Abbott Laboratories, Chicago,
Results

Physical Characteristics of RIF. It was initially observed that RIF activity, present in RIF+ sera, could be effectively segregated from serum proteins by a single analytical flotation at 189,000 g. RIF was recovered in the low-density fraction (<1.10 g/cm³), and this fraction was enriched in lipoproteins of beta (β) and prebeta (pre-β) electrophoretic types (Table I) consistent with enrichment in LDL and VLDL. Immunelectrophoretic analysis of RIF+ fractions 1 and 2 revealed a single serum protein arc with beta mobility; and a line of identity was observed in gel double diffusion with the single precipitin line formed between serum and anti-β-lipoprotein. HBsAg was absent from the low-density RIF+ fractions and was recovered in fraction 6 with the majority of serum proteins.

Single analytical flotation followed by isopycnic banding of RIF+ sera or serum pools led to recovery of RIF in fractions of densities of 1.050 to 1.059 g/cm³ (Fig. 1). The distribution of RIF activity was not proportional to the distribution of protein, but was recovered in the lower density portion of the LDL fraction. Equivalent fractions from RIF- sera were devoid of RIF activity.

Standard classes of lipoproteins were isolated from RIF+ and RIF- serum by sequential lipoprotein flotation. RIF activity was recovered only from the LDL and VLDL fractions of RIF+ sera. These fractions were pooled, adjusted to a density of 1.050 with KBr, and subjected to isopycnic density gradient ultracentrifugation; RIF activity was recovered in the first run over a range of 1.039 to 1.062 g/cm³. A second isopycnic banding led to improved resolution, and RIF activity was completely recovered with a restricted density of 1.050±0.004 g/cm³ in KBr. Proteins other than β-lipoproteins were not demonstrable by lipoprotein electrophoresis and immunelectrophoresis.

Isolation of RIF. The uniquely restricted density of RIF was exploited to isolate RIF-enriched lipoprotein from 200-ml samples of RIF+ serum or control RIF- serum. After three sequential isopycnic bandings fractions with densities between 1.046 and 1.054 g/cm³ free of contaminating VLDL, high-density lipoprotein (HDL) and conventional serum proteins were pooled and fractionated by ion exchange chromatography on DEAE Bio-Gel. Protein was eluted in a stepwise fashion with increasing concentrations of KBr varying from 0.01 M to 1.0 M. Two broad protein fractions were recovered (Fig. 2). RIF activity was eluted only between 0.02 and 0.04 M KBr, the frontal aspect of the first peak, a minor proportion of the total recovered LDL. Equivalent protein fractions were recovered from the RIF- serum but were devoid of RIF activity.

LDL of 1.046–1.054 g/cm³ was heterogeneous with regard to size when subjected to analytical molecular exclusion chromatography on A-5 agarose columns. A minor peak approximating the void volume possessed RIF activity, whereas the major included peak was devoid of detectable RIF activity. When RIF+ fractions recovered from preparative ion exchange chromatography were subjected to molecular exclusion chromatography, RIF activity was again recoverable at the first, and only detectable, protein peak (Fig. 3). The volume of elution coincided with typical normal LDL (estimated mol wt of 3.5 × 10⁶) (17).
### Table I

**Ultracentrifugal Flotation of RIF**

| Fraction | RIF | HBsAg $^\dagger$ | **Serum proteins present** |
|----------|-----|-----------------|----------------------------|
|          |     |                 | Serum proteins present     |
|          |     |                 | Lipoprotein                  |
|          |     |                 | electrophoresis             |
|          |     |                 | Immuno-                      |
|          |     |                 | electrophoresis             |
| 1        | 32  | 0               | $\beta$, pre-$\beta$        |
| 2        | 30  | 0               | $\beta$                     |
| 3        | 0   | 0               | $\beta$, $\alpha$          |
| 4        | 0   | 0               | $\beta$, $\alpha$          |
| 5        | 0   | 1+              | $\alpha$                   |
| 6        | 0   | 4+              | albumin + other proteins    |

$^*$ Single analytical flotation at 189,000 g in KBr of a RIF$^+$ serum pool.

$^\dagger$ Expressed as 1–4$^+$, where 4$^+$ is equivalent to serum pool.

---

**Fig. 1.** Single analytical isopycnic banding of the RIF$^+$ lipoprotein fractions of serum previously subjected to a single analytical flotation. The sample was adjusted to 1.063 g/cm$^3$ and centrifuged at 114,000 g for 72 h at 20°C. RIF activity was recovered only from the lower density (1.050–1.059 g/cm$^3$) portion of the major LDL band (1.050–1.070 g/cm$^3$).

This material, prepared from a single 200 ml high titer RIF$^+$ serum, was concentrated to 2 ml, a protein concentration of 20 μg protein/ml.

**Dose-Response Characteristics and Recovery of RIF.** The quantitative recovery of RIF activity from starting serum was evaluated by RIF assays employing serial dilutions of purified RIF and starting serum. The maximum absolute inhibition produced by the serum and the purified RIF was identical, the dose responses were parallel, and both produced approximately 50% absolute inhibition of the E rosette positive cells relative to saline and normal control serum. Results were expressed as percent of maximum attainable inhibition (Fig. 4). 50% relative inhibition was produced by the purified RIF preparation at a dilution of 1:6,561, equivalent to a concentration of 170 pg protein in the sample and 690 pg protein/ml final concentration in the cultures. The use of RIF$^-$ serum rather than PBS or fetal calf serum as diluent had no effect on the dose response curve. Based on an average mol wt of $3.5 \times 10^6$ and 20% protein for LDL (17, 18),
FIG. 2. Fractionation of partially purified RIF by analytical ion exchange chromatography on DEAE-Bio-Gel. An aliquot of RIF+ LDL of density 1.050±0.004 g/cm³ in 0.005 M Tris-buffer pH 8.3 was applied to a column in the same buffer. Stepwise elution was carried out with increasing concentrations of KBr. RIF activity was demonstrated only in the 0.02 M–0.04 M KBr eluates.

FIG. 3. Molecular exclusion chromatography on Bio-Gel A-5 of partially purified RIF from ion exchange chromatography. A single protein peak close to the void volume of the column and equivalent to that of normal LDL is demonstrated. RIF activity is restricted to the protein peak.

an estimated concentration of purified RIF required for 50% inhibition is approximately $1 \times 10^{-12}$ M in culture. Based on protein recovery, isolation of RIF involved a 300,000-fold purification from serum. The ratio of the reciprocals of the dilutions of purified RIF and original serum required for 50% RIF activity indicated a 436% recovery of RIF activity.

**Immunochemical Characterization of RIF.** The presence of RIF activity on individual human serum proteins was analyzed in double antibody immunoprecipitation assays. Purified RIF and the original RIF+ serum were diluted in PBS or normal (RIF−) human serum at 20 ng protein/ml and 1:3, respectively.
Incubation of these samples with antisera to human albumin, immunoglobulins, alpha-1 antitrypsin, HBsAg, HbcAg, transferrin, haptoglobin, or fibrinogen and subsequent precipitation with second antibody was associated with no loss of RIF activity. In contrast, incubation with antibeta lipoprotein completely removed RIF activity from both purified RIF and RIF+ serum.

Similar experiments were performed with antisera to each of the human apolipoproteins. RIF was bound by antisera to beta-lipoproteins and to apoproteins A_{II}, B, and C_{III}, but not by antisera to apoproteins A_{I}, C_{I-II}, or D (Table II). The antigenic determinants of both alpha lipoproteins and beta lipoproteins, LP-A and LP-B, were demonstrated by Ouchterlony analysis of the RIF preparation as well as in its biologically inactive counterpart from normal serum. Furthermore, antisera prepared to each of these isolated substances give reactions of identity with antisera reactive with LP-A and LP-B. This body of evidence suggests that RIF activity is expressed either by an association complex of different classes of lipoproteins or by a unique hybrid lipoprotein possessing each of these selected protein chains. The presence of similar apoproteins in the biologically inactive lipoprotein fractions from normal serum suggests that RIF activity may be due to conformational properties of the complex or other structural aspects that remain to be elucidated.

Kinetics of RIF Effect In Vitro. Sera from six RIF+ and five RIF− individuals were used to characterize the temporal kinetics of the effect of RIF on E rosette function (Fig. 5). No effect of five of the six RIF+ sera was observed before 4 h of incubation with the lymphocytes. RIF activity reached a peak at 24 h and remained relatively unchanged during the following 24 h. One of the six RIF+ sera differed in that it exerted a significant inhibitory effect as early as 2 h after incubation; however, maximal inhibition was not observed until 24 h.

Dilution of this latter serum 1:5 with PBS led to an initial 4 h lag period and a 24 h peak effect similar to the curves obtained with the other five RIF+ sera. No serum thus far tested has exerted more than a 40–50% inhibition of absolute E rosette formation regardless of the duration of incubation, time required for initial inhibition, or absolute concentration of RIF.

Lymphocyte Recovery. The time required for lymphocytes to recover from RIF inhibition of E rosette function was evaluated in vitro. After 24 h incubation
**Table II**

*Immunocompatability Analysis of the Apolipoprotein Composition of RIF*

| Antiserum | RIF activity bound* |
|-----------|---------------------|
|           | PBS diluent† | NHS diluent‡ |
| Anti-A₁   | 7.7%         | 5.1%        |
| Anti-A₂   | 100%         | 100%        |
| Anti-D    | 100%         | 100%        |
| Anti-C₁₂  | 0%           | 100%        |
| Anti-C₁₃  | 95.8%        | 6.3%        |
| Anti-D    | 2.6%         | 0%          |
| NRS§      | 0%           | 0%          |
| NGS§      | 0%           | 0%          |

*Calculated from 100% percent supernatant RIF.
†Purified RIF preparation at 20 ng protein/ml.
§Normal rabbit serum (NRS) or normal goat serum (NGS).

---

**Fig. 5.** Temporal kinetics of functional defectiveness of lymphocytes in respect to E rosette function. Six RIF⁺ (-----) and five RIF⁻ sera (-----) are illustrated, and results are expressed as percent relative inhibition at various intervals in culture. The accelerated effect of high titered RIF⁺ serum (●) is normalized by dilution 1:5.

---

with RIF at 4 ng protein/ml, the lymphocytes were washed, returned to culture in the absence of RIF, and assayed for E rosette function at various periods thereafter (Fig. 6). Partial recovery was observed as early as 1 h after washing, proceeded in a linear fashion, and was complete at 6 h. It appears that the continued presence of RIF may be required for functional inhibition of E rosette function.

**RIF Binding to Lymphocytes.** The binding of RIF at 20 ng protein/ml was examined by assay of residual free activity after 1 h incubation. Binding of RIF occurs rapidly and was observed as early as 15 min after exposure to lymphocytes and appeared complete by 1 h. RIF was bound by lymphocyte preparations and the quantity bound was directly proportional to the number of cells (Fig. 7) with a correlation coefficient $r = -0.99$. The number of receptor sites for RIF
FIG. 6. Temporal kinetics of recovery of lymphocytes from RIF-induced inhibition of E rosette function. Lymphocytes were incubated in complete culture media containing purified RIF (4 ng protein/ml) for 24 h. The lymphocytes were washed and returned to culture in the absence of RIF, and E rosette function was assayed at various intervals. Results expressed relative to percent of maximum inhibition observed at 0 h.

FIG. 7. The binding of RIF by lymphocytes. In the top panel an aliquot of purified RIF at 20 ng protein/ml was incubated with varying numbers of lymphocytes for 1 h at 37°C in a vol of 100 µl. Percent of RIF bound was calculated from the quantity of residual free RIF activity of the supernates. In the lower panel the number of receptors for RIF was estimated from saturation studies. Binding was calculated as molecules RIF/lymphocyte and plotted against the bound/free ratio. Least square matched linear regression analysis exhibits good agreement of points \( r = -0.98, P < 0.01 \) with a saturation level of 2,900 molecules of RIF/lymphocyte. An equal number of receptors can be postulated on the basis of a unimolar ratio of RIF and receptor.

was estimated from binding profiles by plotting molecules RIF bound/cell against the bound/free ratio of RIF activity (Fig. 7). Four point analysis provided a straight line linear regression \( r = -0.98, P < 0.01 \) and an estimated 2,900 receptor sites per cell based on an estimated mol wt of \( 3.5 \times 10^6 \) and a 20% protein composition of RIF. The mean binding constant \( k \) estimated at 50% binding of RIF by \( 5.2 \times 10^5 \) lymphocytes under these conditions is approximately
ROSETTE INHIBITORY FACTOR

TABLE III

Effects of RIF on the Mixed Lymphocyte Reaction

| Test substance  | Concentration | cpm     | %  |
|----------------|---------------|---------|----|
| Fetal calf serum | 20%           | 21,782±618 | 100|
| PBS            | 20%           | 17,683±590 | 81 |
| RIF<sup>+</sup> serum | 20%         | 11,495±578 | 53 |
| Purified RIF<sup>+</sup> | 180 ng protein/ml | 1,333±68 | 6  |
| Purple RIF<sup>-</sup> | 180 ng protein/ml | 20,186±643 | 93 |
| RIF<sup>-</sup> serum | 20%           | 24,887±721 | 114|
| Purified RIF<sup>-</sup> | 180 ng protein/ml | 1,583±92 | 7  |

9.3 x 10<sup>10</sup> l/mol, well in accord with a specific interaction between RIF and a specific lymphocyte surface receptor.

Effects of RIF on Other Parameters of Lymphocyte Function. RIF<sup>+</sup> serum was mildly inhibitory in the two-way lymphocyte reaction when compared with normal RIF<sup>-</sup> serum (Table III). However, both purified RIF and the equivalent preparation from RIF<sup>-</sup> serum at 180 ng protein/ml (approx. 2.6 x 10<sup>-8</sup> M) were highly inhibitory and to an equal degree. At 2.6 x 10<sup>-9</sup> M neither preparation induced a significant inhibition of thymidine incorporation. Neither purified preparation nor the original RIF<sup>+</sup> and RIF<sup>-</sup> sera at equivalent concentrations inhibited thymidine incorporation of lymphocytes after stimulation by PHA.

Discussion

In the present study, we have isolated and characterized a new immunoregulatory factor found in sera from patients with hepatitis B virus infection and demonstrated that it is a discrete and unique serum beta lipoprotein. This factor, RIF, modulates the capacity of human T lymphocytes to form rosettes with E, a functional marker of these cells. The pathological association of RIF with chronic hepatitis after hepatitis B virus infection suggests that RIF may influence immunological aspects of tissue injury associated with this viral infection. RIF is not restricted to this viral infection and may influence the pathogenesis of other diseases as well. 4 Although RIF is identified by reference to its capacity to modulate a specific T lymphocyte function in vitro, it also appears to be active in vivo since: (a) T lymphocytes functionally deficient in E rosette capacity occur in the peripheral blood of patients whose serum contains RIF, and (b) when these lymphocytes are incubated in the absence of autologous RIF<sup>+</sup> serum, they reacquire normal E rosette function (9). 3

Because of its low density, RIF can be readily distinguished by simple ultra-

---

4 Curtiss, L. K., and T. S. Edgington. Unpublished observations.
centrifugation from most other serum constituents shown to influence lymphocyte function in vitro including immunoglobulins (19), corticosteroids (20), immunoregulatory alpha globulins (21), glycoproteins (22), and C-reactive protein (23). RIF activity is found at a density less than 1.10 g/cm³ which contains primarily lipoproteins of LDL and VLDL classes. In contrast to the general class of LDL with a density of 1.006–1.063 g/cm³, RIF has a restricted density of 1.050±0.004 g/cm³, and it constitutes only a very minor component of serum LDL. When preparations obtained by repeated sequential isopycnic density gradient ultracentrifugation were subjected to ion exchange and molecular exclusion chromatography, the size of RIF was indistinguishable from LDL with an estimated mol wt of $3.5 \times 10^6$ (17), but was restricted to a weakly anionic subset of these molecules. These observations suggest that RIF activity is associated with a unique species of LDL distinct from the general population of molecules of this lipoprotein class.

Isolation of RIF was associated with recovery of approximately four times more activity than initially present, suggesting that the increased RIF activity results from either modification of an inactive form or the presence of inhibitors. RIF may be present not only in an active form but also in a precursor form that is activated by mechanisms such as modulation of tertiary or quaternary structure during isolation. Alternatively, RIF might be damped in whole serum by the presence of competitive inhibitors or agents independently capable of augmenting E rosette function. Inhibitors were not demonstrable in normal serum; however, their presence in RIF+ sera cannot be excluded.

Evidence to support the former hypothesis is provided by immunochemical assays which demonstrate that the biological activity of RIF is directly associated with and must be resident on molecules containing three different apolipoproteins: A, B, and C. This suggests that RIF activity may be due to either certain properties generated by the association of these three lipoproteins into a lipoprotein complex or the formation of a hybrid species of lipoprotein. Lipoprotein association complexes have been described in normal human serum (24) but usually occur at densities less than 1.019 g/cm³. Because of its unique density we favor the concept that RIF is an unusual hybrid lipoprotein containing apolipoproteins of the A, B, and C classes.

The extraordinary functional potency of RIF supports the specificity of its effect and is consistent with its in vivo activity. Employing reasonable assumptions concerning molecular weight, lipid:protein ratio, and purity of preparations it is estimated that biological activity is observed at RIF concentrations as low as $1 \times 10^{-12}$ M. Saturation studies suggest the availability of approximately 2,900 receptors sites per cell and a binding constant ($k$) of approximately $9.3 \times 10^9$ liters/mol. The high affinity of this interaction suggests that the recovery of lymphocytes from the influence of RIF does not reflect removal of bound RIF, but rather results from removal of available "free" RIF. This conclusion in turn suggests that RIF must be irreversibly utilized in exerting its biological effect. The recovery rate of lymphocytes from RIF indicates a functional half-life of approximately 1½ h. This remarkable potency and rapid turnover underscores the biological significance of this factor.

The capacity of lipoproteins to regulate cellular metabolism has recently been
documented by Goldstein and Brown (25). They have demonstrated the existence on human fibroblasts of a cell surface receptor required for binding of plasma LDL. It appears that the regulation of the sterol content of cells, including the rates of uptake, esterification, and synthesis of cholesterol is modulated by the interaction of LDL with this receptor. The effect on cholesterol biosynthesis appears to follow inhibition of the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate limiting enzyme in the cholesterol biosynthetic pathway (25).

The biological activity of RIF apparently depends upon binding to the lymphocyte followed by initiation of the events that subsequently lead to inhibition of E rosette function. Despite rapid binding, more than 4 h is required for reduction in the number of observed E rosette positive lymphocytes, and maximal effect is not observed for 18–24 h. This temporal sequence suggests that RIF inhibits E rosette function by modification of metabolic events rather than by direct steric hindrance at the lymphocyte surface. The continued presence of RIF, requisite for its biological effect, is also well in accord with a similar active regulatory role.

The functional expression of the RIF effect may be dependent on modification of the rate of synthesis, membrane incorporation, or loss of E receptors. Since (a) the binding of a variety of other proteins to lymphocyte surface membranes is known to activate the adenyl cyclase-cyclic AMP system; and (b) elevated intracellular cyclic AMP levels inhibit E rosette function (2, 3), as well as other lymphocyte functions (26–28), modulation of the cyclic nucleotides system might be responsible for the biological activity of RIF.

RIF appears to inhibit E rosette function of only a subpopulation of T lymphocytes, since greater than 40–50% relative inhibition of E rosette function has not been observed, even with a 1,000-fold excess of RIF. The number of lymphocytes capable of forming "active" rosettes according to the method of Wybran and Fudenberg (6) is normal in RIF+ patients whereas the total number of E rosette positive lymphocytes is reduced. This suggests that lymphocytes that require a longer incubation period for the full expression of E rosette capacity are the cells that are inhibited by RIF. Whether this relates to density of receptors for E, lower affinity of receptors, availability of receptors for RIF, or susceptibility to inhibition remains to be established.

The available evidence suggests that RIF may represent an abnormal association complex or a hybrid form of lipoprotein incorporating an unusual set of apolipoproteins. Since these same species exist separately and in various association complexes in RIF+ sera, the biological properties must be attributed to the nature of the complex rather than the apolipoproteins per se. The role played by the lipid moieties of RIF must be investigated, particularly in light of studies suggesting that fatty acids may influence lymphocyte function (29).

The observation that an abnormal lipoprotein, lipoprotein X, is found in the serum of patients with obstructive jaundice (30) is of interest since it appears to be an abnormal association complex of apolipoproteins, lipids, and other serum proteins. It is possible that hepatocellular injury may induce alterations in hepatocyte biosynthetic pathways that result in the synthesis of normal apolipoproteins which associate with lipids and other proteins in abnormal arrays or...
result in the formation of unusual hybrid molecules such as RIF. Alternatively, RIF may be a normal intracellular constituent of the hepatocyte and may be released into the circulation as a consequence of hepatocellular necrosis. Indeed Schumacher et al. have identified a factor in normal human liver which is capable of inhibiting the response of lymphocytes to phytohemagglutinin and allogeneic cells (31).

Summary

Rosette inhibitory factor, RIF, previously described in serum from patients with hepatitis B virus infection, has been isolated and identified as a minor species of \( \beta \)-lipoprotein of the low-density lipoprotein (LDL) class. It is unrelated to hepatitis B virus proteins or particles. Although discrete by reference to charge and density (1.050±0.004 g/cm\(^3\)), RIF appears to be a complex macromolecular structure containing apolipoproteins A, B, and C. Greater than 400% recovery is achieved upon 300,000-fold purification from RIF\(^+\) sera suggesting activation of a precursor form that is not present in normal serum.

RIF inhibits E rosette function of T lymphocytes in vitro with a lag period of approximately 4 h and maximal effect at 24 h consistent with a metabolically-induced event. RIF is functionally active at concentrations of \( 1 \times 10^{-12} \) M or greater, rapidly binds to lymphocytes, and has a functionally effective half-life of approximately 1.5 h. Approximately 2,900 receptors for RIF appear to be present per cell and a high mutual affinity is apparent \( (k = 9 \times 10^{9} \text{ liters/mol}) \). RIF has no detectable effect on mitogen (PHA) responsiveness of lymphocytes, but inhibits the capacity of lymphocytes to respond to histoincompatible cells in vitro at concentrations greater than \( 10^{-8} \) M. Equivalent RIF\(^-\) lipoprotein fractions from normal serum are equally inhibitory in the mixed lymphocyte reaction suggesting that this effect is not directly attributable to RIF activity.

These data indicate that RIF is a unique and functionally specific species of LDL that represents either an association complex of lipoproteins or a hybrid molecule of unusual composition. The association of this factor with viral-induced hepatocellular injury underscores the need to elucidate its structure and function in greater detail.

The authors thank Dr. J. Routenberg who kindly provided the patient sera, Dr. L. Curtiss for assistance with the mixed lymphocyte cultures, and Dr. P. Alauopovic for the antiserum to specific apolipoproteins. The technical assistance of D. Peterson and J. Gealy and the secretarial assistance of M. Gortmaker is gratefully acknowledged.

Received for publication 16 June 1975.

References

1. Bentwich, Z., S. D. Douglas, F. P. Siegal, and H. G. KunkeI. 1973. Human lymphocyte-sheep erythrocyte rosette formation: some characteristics of the interaction. Clin. Immunol. Immunopath. 1:511.
2. Chisari, F. V., and T. S. Edgington. 1974. Human T lymphocyte "E" rosette function. I. A process modulated by intracellular cyclic AMP. J. Exp. Med. 140:1122.
3. Galant, S. P., and R. A. Remo. 1975. \( \beta \)-adrenergic inhibition of human T lymphocyte rosettes. J. Immunol. 114:512.
4. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J. Exp. Med. 136:207.
5. Bushkin, S. C., V. S. Pantic, and G. S. Incy. 1974. Studies on the mechanism of human peripheral blood lymphocyte receptors formation in vitro. Fed. Proc. 33:629.
6. Wybran, J., and H. H. Fudenberg. 1973. Thymus-derived rosette forming cells in various human disease states: cancer, lymphoma, bacterial and viral infections, and other diseases. J. Clin. Invest. 52:1026.
7. Messner, R. P., F. D. Lindstrom, and R. C. Williams. 1973. Peripheral blood lymphocyte cell surface markers during the course of systemic lupus erythematosus. J. Clin. Invest. 52:3046.
8. Edgington, T. S., and F. V. Chisari. 1975. Immunological aspects of hepatitis B virus infection. Am. J. Med. Sci. 270:213.
9. Chisari, F. V., and T. S. Edgington. 1975. Two mechanisms of null cell generation in a prototype human viral infection. Fed. Proc. 34:1012.
10. Hatch, F. T., and R. S. Lees. 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21(Suppl.):97.
11. Menzoian, J. O., A. H. Glasgow, R. D. Nimberg, S. R. Cooperband, K. Schmid, I. Saporoschetz, and J. A. Mannick. 1974. Regulation of T lymphocyte function by immunoregulatory alpha globulin (IRA). J. Immunol. 113:266.
12. Chase, P. S. 1972. The effect of human serum fractions on phytohemagglutinin and concanavalin A stimulated human lymphocyte cultures. Cell Immunol. 5:544.
13. Mortensen, R. F., A. P. Osmond, and H. Gewurz. 1975. Effects of C-reactive protein on the lymphoid system. I. Binding to thymus-dependent lymphocytes and alteration of their functions. J. Exp. Med. 141:821.
lipoproteins of normal human plasma. Evidence for the occurrence of lipoprotein B in associated and free forms. *Biochem. J.* 137:155.

25. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low-density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* 249:5153.

26. Smith, J. W., A. L. Steiner, and C. W. Parker. 1971. Human lymphocyte metabolism. Effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin. *J. Clin. Invest.* 50:442.

27. Henney, C. S., H. R. Bourne, and L. M. Lichtenstein. 1972. The role of cyclic 3', 5' adenosine monophosphate in the specific cytolytic activity of lymphocytes. *J. Immunol.* 108:1526.

28. Chisari, F. V., R. S. Northrup, and L. C. Chen. 1974. The modulating effect of cholera enterotoxin on the immune response. *J. Immunol.* 113:729.

29. Mertin, J., and D. Hughes. 1974. Specific inhibitory action of polyunsaturated fatty acids on lymphocyte transformation induced by PHA and PPD. *Int. Arch. Allergy Appl. Immunol.* 48:211.

30. Seidel, D., P. Alaupovic, and R. H. Furman. 1969. A lipoprotein characterizing obstructive jaundice. I. Method for quantitative separation and identification of lipoproteins in jaundiced subjects. *J. Clin. Invest.* 48:1211.

31. Schumacher, K., G. Maerker-Alzer, and V. Wehmer. 1974. A lymphocyte-inhibiting factor isolated from normal human liver. *Nature (Lond.)*. 251:655.