L428 Cells Derived From Hodgkin's Disease Produce E Rosette-Inhibiting Factor

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Diminished rosetting capacity of T cells is a well-known phenomenon in Hodgkin's disease, and inhibitors of E rosette formation have been reported to be present in the plasma of patients with Hodgkin's disease. The cell line L428, representing an in vitro counterpart of Hodgkin and Sternberg-Reed cells, could be shown to release a factor capable of suppressing the binding of sheep red blood cells (SRBC) to normal peripheral blood lymphocytes (T cells) at a density of 0.5 to 1 x 10^6 cells/mL. At maximally effective concentrations, RIF (rosetting inhibiting factor) inhibited T lymphocyte rosetting by approximately 40% (mean from 184 healthy controls). The diminished E rosetting of T lymphocytes from Hodgkin's patients was not further suppressed by added RIF. This factor inhibited binding of SRBC to their target cells at 37°C but not at 4°C. The factor could be stored lyophilized at -20°C and was stable at 56°C (30 minutes). RIF was inactive below pH 6 and above pH 9 or after trypsin digestion. Purification by affinity, ion exchange, and molecular sieve chromatography showed activity peaks at 12.5 Kd, 25 Kd, 50 Kd, and 100 Kd.

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Preparation of Supernatant

Culture supernatants of L428 cells (5 x 10^6 cells/mL) grown for 3 days in FCS-free conditions were harvested and centrifuged once at 200g for 15 minutes, twice at 1,700g for 20 minutes. After addition of the protease inhibitor Aprotinin (1000 U/mL; TrasyloL Bayer, Leverkusen, FRG) and adjustment of the pH with HEPES buffer (10 mmol/L, final concentration, Whittaker Bioproducts, Walkersville, MD), the solution was passed through a 0.2-μm filter (Sartorius, Göttingen, FRG) and concentrated at 4°C by ultrafiltration (mol wt exclusion 5 Kd; Amicon, Danvers, MA). Unless specified otherwise, the material was then lyophilized.

Rosette Assay for Determination of RIF-Activity

Mononuclear cells (2 x 10^6; PBL; separated by Ficoll-Hypaque [FH] density centrifugation) or L735 were diluted in 0.5 mL RPMI 1640 medium conditioned with HEPEs, penicillin, streptomycin, and glutamine (PSG) and incubated with 0.2 mL of serial dilutions (5^-1 to 5^-30) of test samples. After incubation at 37°C for 60 minutes, the cells were washed once with RPMI 1640, then incubated with 0.5 mL neuraminidase-treated SRBC (sheep red blood cells) for 5 minutes at 37°C, centrifuged at 200g for 5 minutes, and incubated overnight (16 hours) at 4°C. After addition of Acridine Orange, the percentage of E rosette-forming cells was determined with a Leitz fluorescence microscope (Oberkochen-Württemberg, FRG).

Chromatography

Hydroxyapatite chromatography. Lyophilized supernatant was dissolved in 10 mL starting buffer (1 mg protein/mL) and applied to a Hydroxyapatite column/FPLC (18 x 2.6 cm, BioRad, Richmond, CA), and fractionated by a linear phosphate gradient (phosphate buffer 0.01 mol/L to 0.6 mol/L; pH 7.4; retention volume, 700 mL; flow rate, 1 mL/min).

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Submitted February 9, 1989; accepted April 21, 1990.

Supported by grants from "Deutsche Krebshilfe e. V." and "Deutsche Forschungsgemeinschacht." Address reprint requests to Idiko Katay, MD, Medizinische Klinik I, der Universität zu Köln, Joseph-Stelzmann-Str, 9, D-5000 Köln 41, West Germany.

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791
Amino Acid Analysis

Lyophilized protein tested positive for activity was diluted in 10 mL of starting buffer (2 mg protein/mL 0.01 mol/L Tris-HCl, pH 8.0) and separated on a Mono Q anion exchange column/SMART (1 mL; Pharmacia, Uppsala, Sweden) with a linear NaCl gradient (NaCl 1 mol/L; retention volume, 60 mL; flow rate, 1 mL/min).

Molecular sieve chromatography. Lyophilized supernatant was dissolved in 4 mL starting buffer for separation on a Bio-Gel A 0.5 column (120 x 2.6 cm, BioRad), 120 mg protein/mL 0.1 mol/L phosphate buffer, pH 7.2, flow rate 17.5 mL/min, and on a LKB TDK 4000 Blue Column/HPLC (Bromma, Sweden), 30 mg protein/mL 0.2 mol/L phosphate buffer, pH 7.2, flow rate 0.5 mL/min.

Protein electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been performed with the Phastsystem (Pharmacia).

Amino Acid Analysis

Amino acid analysis has been performed with an analyzer from Applied Biosystems 420A by G. Multhaup at Professor K. Beyreuther's laboratory, ZMBH, Heidelberg, FRG.

Fluorescence-Activated Cell Scanner Analysis (FACSscan)

Fluorescence-activated cell scanner (FACS) studies have been performed with a FACScan (Becton Dickinson, Mountain View, CA). T11,1A (10C7E9) and T11,1B (539/8a) were a kind gift of Professor S. Meuer (DKFZ, Heidelberg, FRG). T11,1B (OKT1 1) has been purchased from Ortho-mune (Raritan, NJ). Antibodies against CD4 (Biotin-IOT4) and CD8 (Biotin-IOT8) were purchased from Immunotech Laboratories (Marseille, France), magnetic bead coupled antibodies using a sorting column (Bergisch Gladbach, FRG).

Statistical Evaluation

The paired Students t test has been used to calculate the statistical significance of the given data. A deviation of more than 30% from the control values has proved to be a reliable criterium for practical purposes.

RESULTS

RIF Production by Different Cell Lines

Cell-free supernatants of the Hodgkin cell lines L428, L428KS, L428KSA, L540, and L591 grown under serum-free conditions were harvested at day 3 and tested for RIF activity. The supernatant of L428 provided highest activity compared to other Hodgkin cell lines (L428KS, L428KSA, L540, L591). For that reason L428 supernatants were chosen for factor purification. Serum-free supernatant of L428 was tested for the presence of RIF activity at days 1 through 6. Maximal inhibition was obtained at day 3, correlating with the overall viability of the cells in culture.

Determination of RIF Activity

Whereas the native supernatant of L428 inhibited E rosette formation, the same material did not show activity after concentration by Amicon filtration and lyophilization. When the concentrated material was rediluted to starting volume, activity was again demonstrable. In some serum-free supernatants from L428 cultivated from frozen stocks, E rosette inhibition could only be detected after dilution of supernatant. A possible explanation for these observations could be varying factor production. Comparison of our Hodgkin cell lines showed not only highest activity in fresh L428 supernatant but also highest dilutability. The effect of serial dilution of RIF activity is shown in Fig 1. At high- or low-factor concentration, no effect on E rosetting could be detected, whereas in between, significant inhibition was observed. This bell-shaped relationship and the degree of maximal rosette inhibition did not change during further purification, but the activity was found at higher dilutions while total protein content was decreasing. For all the data presented in this report, serial dilutions of RIF have been tested.

Physical Stability of RIF

The half-life of the RIF activity in crude supernatant or partially purified material in solution at 4°C or -20°C was about 3 weeks. Storage at temperatures of 20°C or 37°C caused a total loss of activity within hours. RIF was stable over months if stored at -80°C or as a lyophilized preparation at -20°C. Activity was preserved up to 30 minutes at 56°C but was completely lost after 1 minute at 100°C. RIF was inactivated under mild acidic (pH 6) or alkaline (pH 8) conditions (Table 1).
ROSETTE-INHIBITING FACTOR FROM HODGKIN CELLS

Enzyme Treatment

RIF activity of partially purified supernatant (after ion exchange chromatography) was retained when treated with neuraminidase but did not withstand trypsin incubation.

Chromatographic Purification

Chromatography (Fig 2) of the lyophilized supernatant (starting with 10 L resuspended in 4 mL) on a calibrated Biogel A 0.5 column yielded active fractions at 100, 50, and 12.5 Kd (Fig 3). In a former purification, additional activity could be demonstrated at 25 Kd that could not be detected in the profile shown in Fig 3 due to overload of the column in the 50-Kd range.

Further purification of lyophilized active fractions was performed either on a hydroxylapatite column (elution of active RIF at 0.2 mol/L and between 0.4 and 0.6 mol/L phosphate concentration) or anion exchange column (elution of RIF activity at a NaCl concentration between 0.38 and 0.44 mol/L). Sequential purification by molecular sieve and anion exchange chromatography yielded pure protein, as documented by SDS-PAGE (Fig 4) with RIF activity.

Fig 2. Sequential purification protocol. SN L428, 10 L of serum-free supernatant, Amicon-concentrated, lyophilized and resuspended in 4 mL H2O; kD-pool, combined RIF-active fractions of the indicated mol wt range; MQ, purification by anion exchange chromatography; HA, purification by hydroxylapatite chromatography; +, single protein band after SDS-PAGE with RIF activity of the respective mol wt; −, several protein bands after SDS-PAGE of the indicated mol wt range.

Fig 3. RIF activity in the SN of L428 after passage over Bio-Gel A 0.5; molecular sieve chromatography. Serum-free supernatant yields three activity peaks at 100, 50 and 12.5 Kd mol wt. Serial fivefold dilution curves were performed on all fractions. The Y axis shows the dilution that gave maximal rosette inhibition (greater than 30% to 60%). Only active regions are shown.

Aminoacid analysis of the 12.5-Kd and 50-Kd protein band gave identical results. This strongly supports the suggestion of a monomer of 12.5-Kd with a tendency to aggregation or polymerization. Rechromatography of the 100-Kd peak on a LKB TDK 4000 Blue Column (HPLC) revealed activity in the 50-Kd and 25-Kd fraction. Chromatofocusing showed activity in fractions at pH 7.8 to 8.1.

Biochemical and Biologic Properties

Incubation of sheep or human erythrocytes with RIF did not show a reliable effect on E rosetting, whereas incubation of T lymphocytes reproducibly showed significant inhibition. For that reason activity of a given material (supernatant or column fractions) was tested by incubation of T lymphocytes.

The optimal incubation period for the demonstration of the RIF effect was 60 minutes at 37°C, with less inhibition at 30 minutes and no additional effect up to 180 minutes. Maximum achievable inhibition of healthy donor PBL rosetting was 60%, with a mean of 38% (in 184 healthy controls, P < .001). Using the T-ALL line L735, similar inhibition was demonstrable (mean 35%, n = 64, P < .001). In lymphocytes from patients with active Hodgkin’s disease, the initially diminished E rosetting could not be further suppressed (Fig 5). This impaired rosetting capacity of Hodgkin PBL could be restored by overnight incubation in at least 6% FCS. After FCS treatment of T cells from healthy donors as well as from Hodgkin’s patients, less than 25% rosette inhibition by RIF incubation was observed, which we consider not to be significant. RIF also inhibited the rosetting of peripheral blood T cells and L735 with normal human erythrocytes.

E rosette inhibition by RIF has never been observed to reach 100%. A possible explanation might be an effect specific for some subpopulation(s). To address this problem we tested rosette inhibition on T4+ and T8+ lymphocyte populations from healthy donors. T4+ and T8+ subclasses were purified by overnight adhesion to plastic surfaces to remove monocytes and MACS45 to more than 95% as shown by FACS analysis. The E rosette inhibition was identical for both cell populations in these experiments (data not shown).
No inhibitory effect could be observed when PBL were incubated with RIF at 4°C; however, reincubation of this aliquot at 37°C fully restored inhibition. Addition of FCS (end concentration 6%) abolished inhibitory activity.

To study the possible effect of RIF on the CD2 receptor, blocking experiments with RIF and antibodies against the T11,1A epitope of the CD2 molecule have been performed. T11,1A is known to be the binding site of T11TS, the CD2-ligand structure on SRBCs. However, staining with T111A (10C7E9) and T11,1B (539/8a, OKT11), antibodies did not detect differences (either at 37°C or at 4°C) in expression of this epitope after RIF incubation of the cells as shown by FACS scanning. Further experiments are needed to investigate possible RIF effects on the other functional epitopes of CD2 so far described (T11,1A', T11,2A, T11,2A', T11,2B).

DISCUSSION

In Hodgkin's disease (HD) diminished E rosetting capacity of T lymphocytes is a well-known phenomenon.\(^{27,28}\)

Inhibitors of E rosette formation have been reported to be present in the plasma of patients with active HD.\(^{14-16,29}\) Holm et al\(^{14}\) and Sudgen et al\(^{29}\) demonstrated that the inhibitory activity of HD sera was located in the lipoprotein fraction of plasma, confirming earlier observations of Fuchs et al\(^{20}\) and Bieber et al.\(^{17}\)

L428, a cell line representing an in vitro counterpart of Hodgkin and Sternberg-Reed cells, produces a factor capable of diminishing the rosette-forming capacity of T lymphocytes with SRBC as well as with human erythrocytes (rosette-inhibiting factor = RIF).

Similar to results of other investigators who have purified rosetting-inhibitory factors from serum in patients with chronic renal failure,\(^{30}\) hepatitis B,\(^{31}\) or early breast cancer,\(^{12}\) we found up to 60% inhibition of rosette formation.

The diminished rosetting capacity of PBL from Hodgkin's patients is not further decreased by exogenously added RIF. Whether this is due to the presence of "endogenous RIF" in the plasma of Hodgkin's patients remains to be proven. After

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**Fig 4.** SDS-PAGE (8% to 25%; Phastsystem, Pharmacia). Lane 1, mol wt standard SDS 8H (Sigma); lane 2, cell supernatant of L428; lane 3, RIF-active pool of Bio-Gel A 0.5 fractions of the 100-Kd mol wt range (B100); lane 4, 50-Kd protein after passage of B100 over MonoQ column (B100-M50); lane 5, free lane; lane 6, RIF-active pool of Bio-Gel A 0.5 fractions of the 25.6 Kd mol wt range (B12.6); lane 7, 25.6 Kd protein after passage of B12.6 over MonoQ column (B12.6-M12.6); lane 8, mol wt standard SDS 17 (Sigma).

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**Fig 5.** Comparison of RIF action on T cells of different origin (healthy donors, Hodgkin's patients, T-ALL line L735) with purified or partially purified protein. No further suppression of diminished E rosetting capacity of T lymphocytes of Hodgkin's patients by RIF can be seen. Serial fivefold dilution curves were performed in all experiments. The Y axis shows percent rosette formation (for RIF, the % rosettes at the maximally active dilution). The error bars indicate the interindividual or, for L735, the interexperimental variation (±1 SD). The P values refer to a paired t test to evaluate the significance of the RIF-inhibitory effect.
overnight incubation of HD-PBL in FCS, their impaired rosetting capacity returns to normal, but treatment of these cells with RIF results in less than 25% E rosette inhibition, which is not significant. PBL from healthy donors, pretreated with FCS in the same way, also show only 25% rosette inhibition by RIF. The FCS effect cannot be overcome by simple washing. Human serum from healthy donors does not interfere with RIF activity, nor does overnight incubation with this serum restore the impaired rosetting of T cells from Hodgkin’s patients.

RIF action appears to be dependent on cell metabolism, since activity is demonstrable after incubation of lymphocytes at 37°C but not at 4°C. RIF can be stored as a solution at -80°C or after lyophilization at -20°C. Other conditions such as freezing at -20°C and storage at 4°C, 25°C, and 37°C lead to rapid total loss of activity. RIF is labile at unphysiologic acid and alkaline pH, resists heat treatment up to 56°C for 30 minutes, but is destroyed at 100°C. It is trypsin sensitive.

Partial purification by affinity, ion-exchange, and molecular sieve chromatography eluted activity in the 100-Kd, 50-Kd, 25-Kd, and 12.5-Kd fraction, suggesting an oligomer of possibly identical subunits. This assumption is supported by identical aminoacid analysis for the 50-Kd and 12.5-Kd protein band and the disaggregation of the 100-Kd material into active fractions at 50-Kd and 12.5-Kd after rechromatography.

Activity of the factor follows a bell-shaped dose response relationship. Similar dose response curves have been reported for some growth factors and lectins.

Functional studies did not show any identity with other cytokines produced by the Hodgkin cell lines, such as colony-stimulating activity (CSA), interleukin-1 (IL-1), Hodgkin-derived leucocyte factor (HDLF), or Hodgkin-derived growth factor (HDGF). These other factors differ in mol wt and have been shown not to inhibit E rosetting. Other molecules that have been reported to suppress E rosetting of T lymphocytes such as oxygenated sterols, ferritin, and ceruloplasmin are of higher prc. of lower mol wt. RIF activity could not be extracted by low-density lipoprotein (LDL) separation methods but can bind to LDL (data not shown).

Definitive characterization of the described activity by glycopeptidase treatment and protein sequencing is in progress. The receptor and ligand responsible for E rosette formation have been identified and cloned. The human functional analog of the SRBC T11 target structure (LFA 3) has been determined. The CD2 receptor has recently been assigned the role of an alternative pathway of T-cell activation. The binding sites of different MoAbs describe a functional division into two major regions of the CD2 molecule: T11α and T11β. Antibodies to the three epitopes of T11α (T11,1A, T11,1A', T11,1B) interfere with binding of the natural ligand of the CD2 (LFA3/T11TS). The T11β region is not involved in binding of LFA3/T11TS. According to their role in activation of T cells via CD2, three epitopes of T11β (T11,2A, T11,2A', T11,2B) have been identified.

Blocking and activation tests are currently being conducted to identify the exact site of RIF interaction with the CD2 receptor. Up to now, blocking studies with MoAbs have been performed on the T11,1A and T11,1B epitopes of the T11α region of CD2 that mediates E rosetting through binding of the LFA3/T11TS antigen. Our preliminary results show no effect of RIF on T11,1A and T11,1B antibody binding either at 37°C or at 4°C. This may be due to the much higher affinity of antibodies for their target structures as compared to that of the receptor for its ligand, as has been shown for the LFA1/ICAM1 interaction. Dustin and Springer showed an increased adhesiveness of LFA1 to ICAM1 by second-antibody crosslinking of T3Ti (TCR). This was transient and temperature dependent and not associated with a change in cell surface expression as measured by immunostaining.

As upregulation or downregulation of receptor expression or functional modulation by a third activation site cannot be excluded, further experiments on expression and regulation by cell metabolism have to be performed.

Discovery of the physiologic relevance of the CD2 receptor fuels speculation concerning RIF. The receptor is postulated to be the region of influence of RIF and is at the same time a means of T-cell activation, be it alone or in combination with other receptors. If RIF could be demonstrated to interfere with the activation of T cells, this could help to explain one step in the pathogenesis of the functional T-cell defect in Hodgkin’s patients.

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L428 cells derived from Hodgkin's disease produce E rosette-inhibiting factor

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