ANTIBODIES IN HUMAN SERA TO ONCORNAVIRUS-LIKE PROTEINS FROM NORMAL OR LEUKEMIC MARROW CELL CULTURES*

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There is increasing evidence that oncornavirus-like information is present in human cells. The bulk of this evidence rests upon reverse transcriptase studies (1–3), DNA-RNA hybridization homologies (4, 5), immunochemical analysis of viral-like antigens in human tissues (6–8), and the isolation of C-type viruses from human tissues (9–11). Recently, Mak et al. (12) and Vosika et al. (13) reported that some human marrows in culture-released particles with some physical and biochemical properties resembling those of oncornaviruses. We have undertaken to examine the immunological properties of particles obtained in similar marrow culture supernates, particularly with regard to (a) whether they contain determinants cross-reactive with mammalian RNA tumor viruses and (b) whether antibodies to such determinants occur spontaneously in human sera. Internally radiolabeled material from marrow culture supernates was used as antigen in a double-antibody immunoprecipitation assay, and precipitated antigens were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. In this paper, we report that proteins similar to those of the major structural proteins of oncornaviruses can be identified in human marrow culture supernates, and that antibodies to these proteins are commonly present in human sera.

Materials and Methods

Patient Material. Bone marrow specimens were obtained from patients with acute myeloblastic leukemia (AML),[1] acute lymphoblastic leukemia (ALL), acute erythroleukemia, chronic granulocyte leukemia (CGL), or nonleukemic patients undergoing hematological investigation. Human sera were aliquoted and frozen at −70°C until use. Pooled normal human IgG and two purified IgG λ-myeloma proteins were generously provided by Dr. B. Underdown, Institute of Immunology, University of Toronto, Toronto.

Viruses. M_4 virus is a mouse leukemia virus spontaneously released by a clone of JLS-V9 cells in culture (14). [^{3}H]leucine and [^{14}C]leucine-labeled M_4 virus was kindly provided by Dr. A. Howatson of the Ontario Cancer Institute.

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[1] *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CGL, chronic granulocytic leukemia; LCM, leukocyte-conditioned medium; RIPA, radioimmunoprecipitation assay; RLV, Rauscher murine leukemia virus; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus; SSV, simian sarcoma virus.

Rauscher murine leukemia virus (RLV) was obtained from Electro-Nucleonics Inc., Fairfield, N. J., and simian sarcoma virus (SSV-1/NC 37) from Litton Bionetics, Kensington, Md.

Marrow Cultures. Nucleated marrow cells from buffy coat preparations were cultured in suspension in α-medium (Flow Laboratories, Inc., Rockville, Md.) for 5–7 days at 37°C in 5% CO_2 in air. Culture media contained 20% fetal calf serum (Flow Laboratories) and 10% leukocyte-conditioned medium (LCM). This LCM, prepared from peripheral leukocytes of a patient with hemochromatosis, has previously been described (3). Before use, fetal calf serum was spun overnight at 100,000 g and then heat inactivated at 57°C for 30 min. Gentamycin (Schering Corporation Ltd., Pointe Claire, Quebec) in a concentration of 100 μg/ml was routinely added to prevent mycoplasma contamination. Cells were cultured in a concentration of 10^6 cells/ml, and generally 5–10 $\times$ 10^7 cells were used per culture.

Radiolabeling of Cultured Marrow Cells. Cultures were labeled continuously with [^{14}C]uridine and either [^{3}H]glucosamine or [^{3}H]leucine using α-medium lacking ribonucleotides alone, or ribonucleotides and leucine. [^{14}C]uridine (540 mCi/mmol) was used at 1 μCi/ml, [^{3}H]glucosamine (12 Ci/mmol) at 50 μCi/ml, and [^{3}H]leucine (58 Ci/mmol) at 50 μCi/ml; all three were purchased from the Radiochemical Centre, Amersham, England.

Preparation of Labeled or Unlabeled Subcellular Fractions. Culture supernates separated from cells by centrifugation at 500 g for 10 min were spun at 12,000 g for 10 min. The resultant pellets were discarded and the supernates sedimented at 100,000 g for 1 h over a cushion of 25% sucrose in TNE (0.05 M Tris, pH 7.5, 0.1 M Nacl, 1 mM EDTA). The sediments were resuspended in 1 ml of TNE, layered over 10 ml of a 20–70% linear sucrose gradient, and centrifuged for 16–18 h at 150,000 g in an SW 41 rotor. 20 fractions were collected, and a 10 μl aliquot from each fraction was counted in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) in a Beckman LS-230 scintillation counter. Fractions of densities 1.20–1.24 g/ml and 1.15–1.19 g/ml were collected in two separate pools, diluted in TNE, and then pelleted at 150,000 g for 1 h. The final pellets were resuspended in TNE. Protein concentration was determined by the method of Lowry et al. (15). Unlabeled marrow isolates were prepared similarly without the addition of radioactivity in the cultures.

Radioimmunoprecipitation Assay (RIPA). The assay was carried out as described by Ihle et al. (16). Labeled marrow material was diluted in TNE to give about 5,000 cpm of ^{3}H or 1,000 cpm of ^{14}C (1–2 μg protein) per 0.05 ml per test. 0.2 ml of serial twofold dilutions of test serum were added and incubated for 1 h at 37°C. Subsequently, 0.2 ml of a 1:2 dilution of goat anti-human IgG (Meloy Laboratories, Springfield, Va.) was added, and the mixture was again incubated at 37°C for 1 h, then at 4°C overnight. All antiglobulins used were tested by immunoelectrophoresis and gave single precipitin lines.

After the overnight incubation, supernates were separated from precipitates by centrifugation at 1,200 g for 10 min and counted in 10 ml of Aquasol. Precipitates were washed three times in a total of 5 ml of TNE and then resuspended in 0.4 ml of TNE and counted in 10 ml of Aquasol. Precipitation was expressed as the percentage of counts in the precipitate relative to the combined counts in the precipitate and first supernate. In a preparation containing both ^{3}H and ^{14}C labels, the percentage precipitation of the ^{3}H counts was the same as that of the ^{14}C counts. As the efficiency of labeling with [^{3}H]leucine/[^{3}H]glucosamine was much higher than with [^{14}C]uridine, dilutions of labeled marrow material were made according to total ^{3}H counts alone.

The maximum precipitable radioactivity varied from one preparation of radiolabeled material to another; the range of variations was from 40 to 70% of input radioactivity. For a given preparation, the titer of a positive serum was equal to the highest serum dilution that caused 50% precipitation of the maximum precipitable radioactivity, and all sera giving 50% precipitation or above were considered positive. For example, for the preparations derived from patient R, which were used for the titration studies reported in this paper, the maximum precipitable radioactivity was approximately 70% of input. Thus, the end point for all sera titrated using these preparations was precipitation of 0.5 $\times$ 70 = 35% of input radioactivity.

Preparation of Immune Precipitates for SDS-Polyacrylamide Gel Electrophoresis. About 60,000 cpm of [^{3}H]leucine- or [^{3}H]glucosamine-labeled marrow material was precipitated by a dilution of serum giving maximum precipitation, as described above. Unbound counts were disrupted by incubating the precipitate for 1 h at 4°C in 0.1 ml of buffer containing 1.2 M KCl, 1% Triton X-100, 0.03 M β-mercaptoethanol, and 0.1 M Tris, pH 7.5. The precipitate was then washed three times in TNE, solubilized in 8 M urea, 1% SDS, and 2% β-mercaptoethanol in 0.1 M Tris, pH 8.6, heated to 56°C for 30 min, and then to 100°C for 2 min before electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis of Labeled Marrow Material and Immune Precipitates   SDS-polyacrylamide gel electrophoresis (7 5% gels) was done by the method of Weber and Osborne (17) Labeled marrow material (about 40,000 cpm) was heated to 100°C for 2 mm in 2% SDS and 2% β-mercaptoethanol before electrophoresis. Immune precipitates were prepared as described above. The gels were run for 3–4 h at a constant current of 3 mA per gel. After fixation in 10% trichloroacetic acid overnight, they were cut into 1-mm slices, eluted in 0 8 ml of a mixture of nine parts of NCS tissue solubilizer (Amersham/Searle Corporation, Arlington Heights, Ill) to one part of water at 50°C for 2 h, and then counted in 10 ml of toluene plus Liquiflour (New England Nuclear).

[^{3}H]leucine-labeled M_4 virus was run in separate gels [^{14}C]leucine-labeled M_4 virus was coelectrophoresed with ^{3}H-labeled marrow material or immune precipitates.

Marker proteins (bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c) were run in separate gels and stained in Coomassie Blue.

Results

Sucrose Density Gradient Analysis of Labeled Marrow Culture Supernates.   To test whether radiolabeled material from marrow culture supernates was associated with particles similar to those reported previously (12), cultures labeled with [^{14}C]uridine and either [^{3}H]leucine or [^{3}H]glucosamine were analyzed by sucrose density gradient centrifugation. Typical results are shown in Fig. 1. [^{14}C]uridine generally peaked at a density of 1.23 g/ml, whereas [^{3}H]leucine or [^{3}H]glucosamine peaked at 1.17 g/ml. A similar pattern of labeling was seen in cultures of 12 AML (9 relapse, 3 remissions), 5 ALL (2 relapse, 3 remission), 2 CGL (relapse), 1 erythroleukemia (relapse), and 7 nonleukemic marrows (1 each from patients with iron deficiency anemia, megaloblastic anemia, benign hyperglobulinemia, bronchogenic carcinoma, prostatic carcinoma, plasmacytoma, and perihemangiocytoma). The only instances of failure to label were two consecutive marrow samples from a patient with chronic lymphocytic leukemia.

In preliminary experiments, reverse transcriptase activity was not observed in culture supernates from marrows cultured in either leucine-deficient or ribonucleotide and leucine-deficient media. Because it was technically difficult to assay for reverse transcriptase activity in culture supernates labeled with ^{14}C and ^{3}H, the enzyme was not assayed in most of the experiments reported in this paper.

RIPA.   To test for the presence in human sera of antibodies against the particles released by cultures of human marrow, radiolabeled material from culture supernates was prepared from appropriate sucrose density regions, and a RIPA was carried out as described in Materials and Methods.

SELECTION OF LABELED ANTIGEN.   In preliminary experiments a comparison was made between labeled material of densities of 1.20–1.24 g/ml and 1.15–1.19 g/ml as antigen in RIPA. With the same positive serum, the higher density material was usually more reactive than the lower density material, in terms of both maximal precipitation and titer. Reaction patterns of the lower density material were often overshadowed by HL-A reactivity, as judged by reactions with sera of known anti-HL-A activity. For this reason, material pooled from the 1.20–1.24 g/ml region was used routinely in the assay.

TITRATION CURVES.   A typical titration curve is shown in Fig. 2. With a constant amount of added anti-IgG (760 μg), most human sera gave maximal precipitation at a dilution of 1:32 or 1:64. Up to a dilution of 1:256 or 1:512,
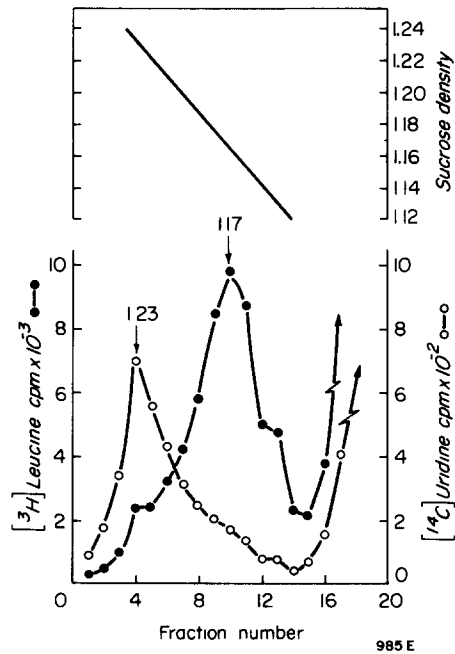
FIG 1. Density distribution of radiolabeled material from marrow culture supernates: (O—O) [¹⁴C]uridine and (●—●) [³H]leucine
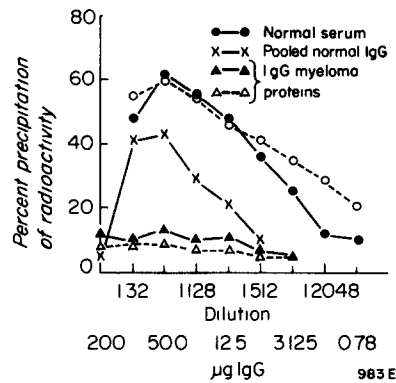

FIG 2. Titration curves of human serum and purified IgG: (●—●) normal human serum, (O---O) 25 μg of IgG myeloma protein added per dilution from 1.256 on; (X—X) pooled normal human IgG; (▲—▲) and (△—△) IgG λ-myeloma proteins.

precipitation was approximately linear with dilution. Beyond this, a carrier protein (one of two purified IgG λ-myeloma proteins, see below) was required to give a straight line end point titration.

SPECIFICITY CONTROLS. To rule out the possibility of nonspecific trapping in the assay, two purified human IgG λ-myeloma proteins were used as controls; myeloma proteins were chosen because of their lack of antibody activity. Both myeloma proteins were negative in the precipitation assay (Fig. 2). These served as negative controls, ruling out any significant nonspecific trapping and were suitable as carrier proteins in titration curves. In contrast, pooled normal
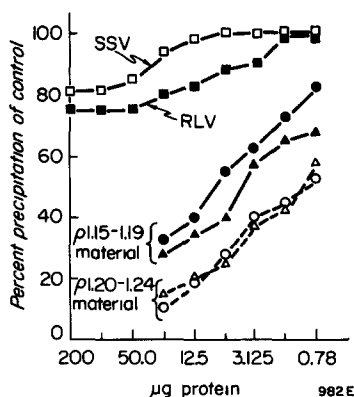
Fig. 3.   Competition in RIPA with unlabeled antigen. Labeled antigen used in the assay was obtained from patient R (AML). In control assays, 50% of total radioactivity was precipitated by serum A.J. at a dilution of 1:256. This dilution of A.J. serum was then preincubated with twofold dilutions of cold antigen (horizontal axis), and the resulting precipitation was expressed as a percentage of the control (vertical axis). Cold antigens used: SSV ($\square$—$\square$), RLV ($\blacksquare$—$\blacksquare$), and unlabeled marrow supernatant material from patient Edi (ALL) ($\bullet$, O) and patient Rus (iron deficiency) ($\blacktriangle$, $\triangle$). Marrow supernatant material of density 1.20-1.24, dashed line; density 1.15-1 19, solid line.

human IgG gave a positive precipitation curve. Thus, it is unlikely that the presence of contaminating materials that are not themselves immunoglobulins can account for the results of RIPAs carried out using unpurified human sera.

A second specificity control was competitive inhibition with unlabeled antigen (Fig. 3). In these experiments, the percentage precipitation of labeled marrow material by a test serum was determined initially. This control value was used as 100% in normalization of data in Fig. 3. The same serum dilution was then preincubated with twofold dilutions of unlabeled marrow material for 1 h at 37°C before RIPA. We used unlabeled marrow material from both 1.20-1.24 g/ml and 1.15-1.19 g/ml density regions in competition experiments. The higher density material was found to contain more competing antigen. Unlabeled material from nonleukemic marrows inhibited precipitation of labeled leukemic marrow material, and the reverse was also observed. These results indicate that the antigen-antibody reaction is specific and is capable of competitive inhibition by similar density material from other marrows. All marrows tested were readily cross-reactive.

Antigenic Competition between Culture Supernates and Known Oncornaviruses.   To determine whether material prepared from human marrow culture supernates contained determinants cross-reactive with mammalian RNA tumor viruses, purified RNA tumor viruses were used as cold antigen to compete in the RIPA. As shown in Fig. 3, when RLV or SSV-1/NC37 was used as competing antigen, much less inhibition was observed, even at high viral protein concentrations, than with unlabeled human marrow material. Similar results were obtained with intact virus or virus disrupted by 10 cycles of freezing and thawing. The extent of cross-reactivity demonstrated in these experiments was slight and at the border of the sensitivity of the method.

Analysis of Precipitated Proteins by SDS Polyacrylamide Gel Electrophore-

sis. Before precipitation the 1.20–1.24 g/ml density marrow material contained a heterogeneous collection of polypeptides (Fig. 4*a*). Upon precipitation with a reactive human serum (Fig. 4*b*) the pattern resolved into one containing three distinct polypeptide peaks of approximately 70,000, 45,000, and 30,000 mol wt, as determined by reference to marker proteins or by coelectrophoresis with labeled M₄ virus (Fig. 4*c*). Similar patterns were obtained from 15 leukemic and 5 nonleukemic marrow culture supernates.

To determine whether any of these polypeptides were glycopeptides, marrow culture material labeled with [³H]glucosamine was precipitated and the immune precipitate analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5, the 70,000 and 45,000 mol wt polypeptides were glycopeptides. These comigrated with the glycoproteins (gp 70 and gp 45) of M₄ virus.

Antibodies in Human Sera as Determined by RIPA. 45 sera from leukemic patients, 45 sera from normal donors, 19 sera from family contacts of leukemic patients, 21 normal cord blood specimens, and 23 sera from patients with systemic lupus erythematosus (SLE) were tested for reactivity against radiolabeled material from culture supernates. Reactivity was demonstrated in a majority of these, and all positive sera precipitated autologous antigen, when available. As shown in Table I, 71% of leukemic sera, 80% of normal sera, 79% of family contacts, and 67% of cord blood specimens had detectable antibody activity. There was no significant difference in the incidence and titer of antibody between leukemic patients in relapse and those in remission (data not shown). 11 leukemic patients over a period of 12 mo (in and out of remission) and 4 normal laboratory workers (2 positive and 2 negative for antibody) over a period of 6 mo showed no change in antibody titer. Lupus patients stood out above the others; 21 out of 23 (91%) had detectable antibody activity. Fig. 6 shows the titers of all sera tested. There is no significant difference between the mean titers of the leukemic, normal, family contact, or cord blood subgroups. However, the mean for the SLE sera is significantly higher than the means of each of the other subgroups on the basis of a *t* test ($P < 0.01$). When the mean titers of only the positive sera were compared, the mean for the positive SLE sera was again significantly higher than the means of the positive sera in the other subgroups ($P < 0.01$).

Discussion

Our results indicate that: (*a*) proteins similar in molecular weight to the major envelope and internal proteins (gp 70, gp 45, p 30) of mammalian C-type RNA viruses can be obtained from normal or leukemic human marrow cell cultures and (*b*) antibodies reactive with one or more of these antigens are found in many individuals, regardless of clinical status. The first observation is in keeping with the findings of Strand and August (7) and Metzgar et al. (8) who demonstrated the presence of gp 67/71 and p 30-like proteins in a variety of diseased and normal human cells. However, our experiments demonstrated only slight, if any, cross-reactivity between these proteins from human marrow culture supernates and murine and simian leukoviruses (see Fig. 3).

It should be noted that while these proteins coprecipitate with material incorporating [¹⁴C]uridine (presumably RNA), we have no additional evidence
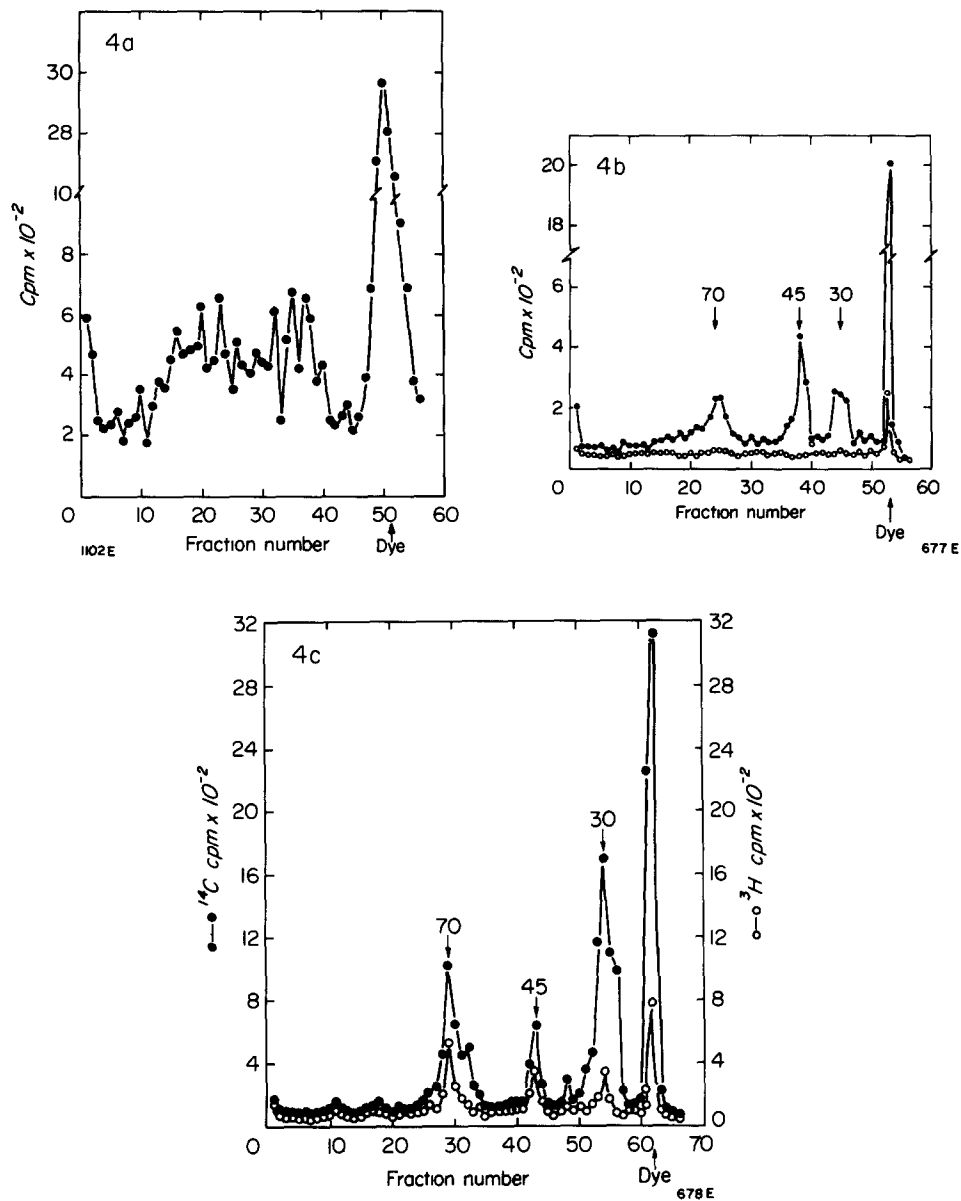
FIG. 4. SDS polyacrylamide gel electrophoresis patterns of [³H]leucine-labeled marrow material from patient Van (ALL): (a) before precipitation; (b) after precipitation by autologous serum (●—●) and negative human serum J.N (O—O); (c) precipitate by autologous serum (O—O) coelectrophoresed with [¹⁴C]leucine-labeled M₄ virus (●—●). Arrows indicate molecular weight in thousands

to indicate that they are associated with virally related particles. It is possible they represent cellular proteins with antigenicities "unmasked" by the culture and preparative procedures. Further work is required to determine if they are truly related to known viral proteins.
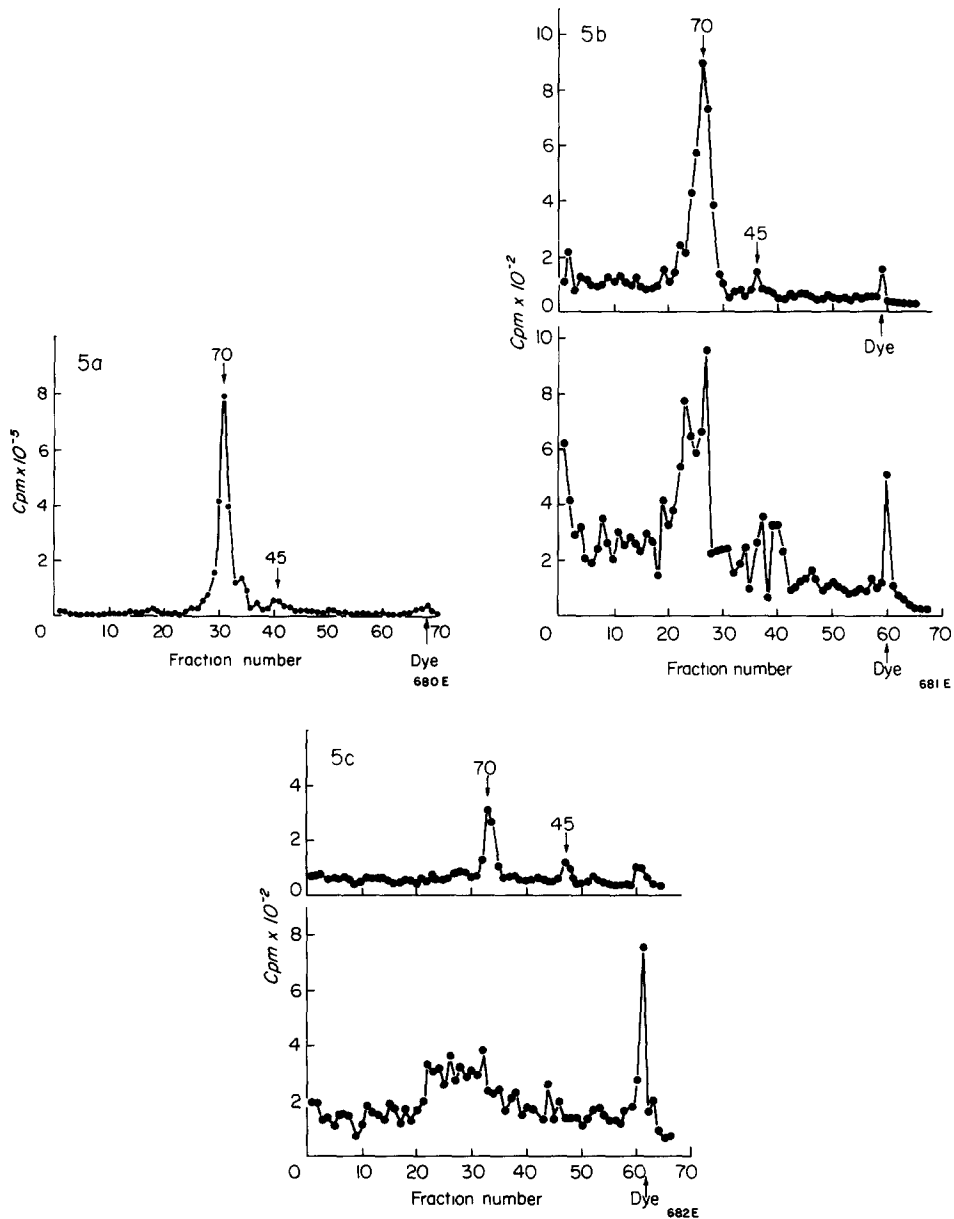
Fig. 5.   SDS-polyacrylamide gel electrophoresis patterns of: (a) [³H]glucosamine-labeled M₄ virus; (b) [³H]glucosamine-labeled marrow material from patient Zaf (AML) before (lower panel) and after (upper panel) precipitation by positive human serum C.C.; (c) [³H]glucosamine-labeled marrow material from patient Rus (iron deficiency anemia) before (lower panel) and after (upper panel) precipitation by serum A.J.

Nonetheless, the presence in human sera of antibodies with specificity for viral-like proteins is not unexpected. Precedence for this phenomenon is seen in mice, in which naturally occurring antibody to murine leukemia virus is detected in virtually all inbred strains (18). Anti-viral antibodies are also detected

TABLE I

The Incidence in Human Sera of Antibodies to RNA Tumor Virus-Like Proteins Obtained From Human Marrow Culture Supernates

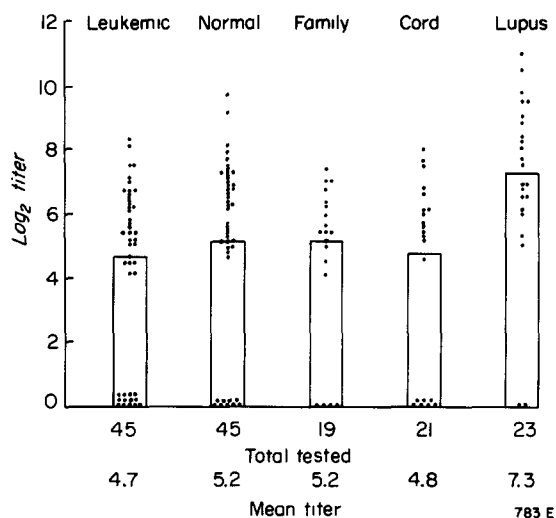| Sera	No. with antibody (total tested)	Percent positive for antibody	Mean titer (Log₂)
Leukemic	32/45	71	4.7
Normal	36/45	80	5.2
Family contacts	15/19	79	5.2
Cord blood	14/21	67	4.8
Lupus	21/23	91	7.3

FIG. 6. Titers of all human sera tested against labeled marrow material from patient R (AML). Family = family contacts of leukemic patients; cord = cord blood specimens; lupus = sera from patients with SLE. Vertical bars represent geometric mean titers of all sera tested.

in cats (19) and gibbons (20); in the latter cases antibodies are more common in colonies with an incidence of leukemia or lymphoma. We have followed 11 leukemic patients over a period of 12 mo, in and out of remission; none of them showed a change in antibody titer. Similarly, four normal laboratory workers showed no change in antibody titer over 6 mo. The relevance, if any, of this virus-like information and immune response to human leukemia is unclear.

Of special interest in this regard are patients with SLE. As a group, they show a significantly higher mean antibody titer. This is in accord with the recent report by Mellors and Mellors (21) of the presence of p 30-like antigen in human lupus kidneys, and may reflect one of the underlying pathogenic mechanisms in this disease.

Summary

Some human marrows in culture release particles with oncornavirus-like properties. This study was designed to examine the immunological properties of

similar particles in human marrow culture supernates. Leukemic and nonleu-
kemic marrows were cultured for 5-7 days in the presence of [^{14}C]uridine and
[^{3}H]leucine or [^{3}H]glucosamine. Labeled supernatant components banding in
sucrose gradient densities of 1.20-1.24 g/ml were used as antigen in a double
antibody immunoprecipitation assay. The assay was validated by end point
titrations and competition with unlabeled antigen; purified myeloma proteins
were used as negative controls. Cross-reactivity with mammalian oncornavi-
ruses, as judged by competitive inhibition of precipitation by these viruses, was
slight and at the border of the sensitivity of the method. Precipitated antigens
analyzed by SDS polyacrylamide gel electrophoresis contained three distinct
polypeptides of about 70,000, 45,000 and 30,000 mol wt; these comigrated with
the gp 70, gp 45, and p 30 of a murine leukemia virus. Similar polypeptides were
obtained from both leukemic and nonleukemic marrow culture supernates. As
determined by the radioimmunoprecipitation assay, 32 of 45 leukemic sera
(71%), 36 of 45 normal sera (80%), 15 of 19 sera from family contacts of leukemic
patients (79%), 14 of 21 cord blood specimens (67%), and 21 of 23 sera (91%) from
patients with systemic lupus erythematosus had detectable antibody activity.

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