Antibodies Produced by Immunization of Goats with 60S Ribosomal Subunits from Novikoff Hepatoma Ascites Cells[1] (35812)

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A number of studies in this and other laboratories have indicated that there are significant differences in the rapidly synthesized nucleolar RNA of tumors and other tissues (1). Initially the newly synthesized nucleolar RNA of tumors was found to have a higher content of cytidylic acid and a lower content of adenylic acid in comparison to corresponding RNA from nontumor tissues. In addition, significant differences have been found in the oligonucleotide frequencies and competitive hybridization of nucleolar and ribosomal RNA of tumors and other tissues (2–5). Since the 28S ribosomal RNA of Novikoff hepatoma ascites cells (NHAC) differed significantly from that of normal liver, it seemed possible that different types of antibodies might be produced to the 60S ribosomal subunits which contain the 28S ribosomal RNA. These findings led to the present experiments in which 60S ribosomal subunits of Novikoff hepatoma and normal liver were used as antigens to determine whether the same or different types of antibodies would be produced.

To this point only a limited number of studies have been made on the development of antibodies to mammalian ribosomes and ribosomal RNA. Dodd and Bigley (6) have shown that antibodies with some specificity for RNA could be formed in response to immunization with rat liver, rabbit liver, and rabbit reticulocyte ribosomes. In addition, Plescia and Braun (7) reported that antibodies could be produced against RNA coupled to appropriate proteins or other carriers. Barbu and Panijel (8, 9) reported that RNA-specific antibodies could be produced by immunizing horses and rabbits with bacterial ribosomes and indicated that these antibodies were effective precipitins for both 30 and 50S subunits of *E. coli* ribosomes. More recently, Noll and Bielka (10, 11) produced antibodies to rat and bovine liver ribosomes in rabbits. Thus far, studies have not been made on the antigenicity of tumor ribosomal subunits although some specificity has been demonstrated for tumor ribosomal proteins, *i.e.*, when Lamon and Bennett (12) utilized ribosomes of plasma cell tumors as antigens, antisera were formed that exhibited specificity for tumor ribosomes as compared to normal rat liver ribosomes or human liver ribosomes in double diffusion assays.

In the present study, agglutinins and precipitins were produced by immunization of goats with tumor 60S ribosomal subunits. These antibodies produce cell aggregation and inhibition of tumor growth presumably because of their binding to ribosomes on or adjacent to the cell membranes (13).

Materials and Methods. Preparation of ribosomal subunits. To obtain the polysomes (3) the livers were excised, minced in several changes of ice-cold buffer containing 0.05 *M* Tris–HCl, pH 7.4, 0.05 *M* KCl, 0.005 *M* $MgCl_2$ and 0.25 *M* sucrose; and homogenized in this buffer in a Teflon–glass homogenizer. After centrifugation for 10 min at 6000*g*, the pellet was separated from the red blood cells and homogenized in a Teflon–glass

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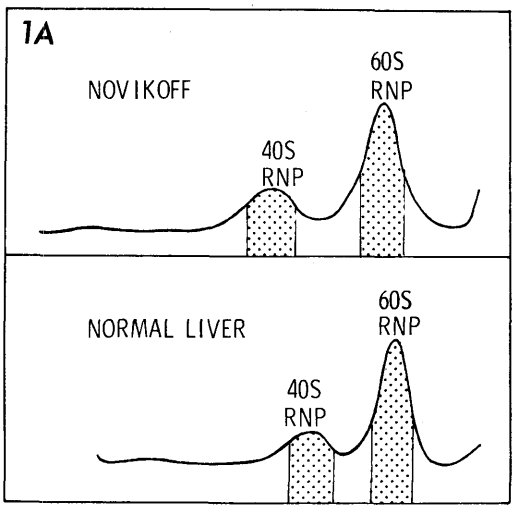

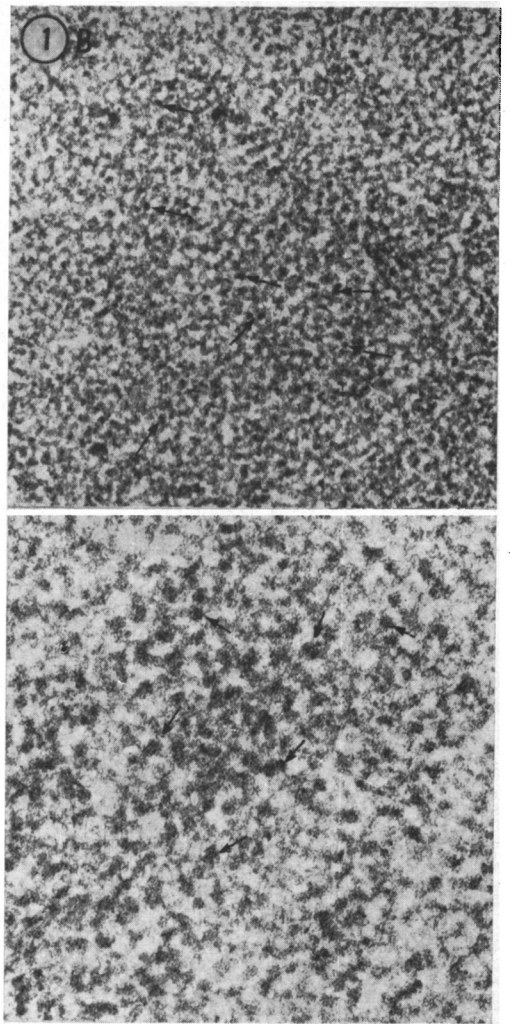

FIG. 1A. Sucrose gradient sedimentation patterns of EDTA-treated polysomes. Conditions are described in Materials and Methods. The fractions in the shaded regions were centrifuged at 240,000*g* for 8 hr and the pelleted 40 and 60S ribosomal subunits were used for both immunization and quantitative analytical studies. (B) Electron micrographs of the 60S ribosomal subunits of the Novikoff hepatoma, isolated as the shaded fraction in (A). Glutaraldehyde-osmium tetroxide fixation, lead citrate-uranyl acetate stain. The purity of these preparations is shown by the fact that no membranous or other subcellular complements were present. (upper) ×69,000; (lower) 123,000. The arrows show the particles and fine details of their organization.

homogenizer with a tight pestle in the same buffer lacking 0.25 *M* sucrose and then centrifuged for 10 min at 12,000*g*. The supernatant was made 0.5% with respect to deoxycholic acid, rehomogenized, and centrifuged for 10 min at 30,000*g*. This supernatant was underlayered with 1/3 vol of 1.0 *M* sucrose containing the above buffer and centrifuged for 16 hr at 78,000*g* in a Beckman No. 30 rotor. To obtain tumor polysomes, the Novikoff hepatoma ascites fluid was centrifuged for 10 min at 6000*g*. The procedure employed for isolation of polysomes from these pelleted cells is the same as that employed for the liver (3).

The pelleted polysomes were suspended in 0.005 *M* Tris–HCl, pH 7.4, with gentle homogenization to give a concentration with an absorbance at 260 mμ of 30 OD/ml. The suspension was made 0.008 *M* with respect to EDTA, layered on a 5–45% sucrose gradient buffered with 0.01 *M* Tris–HCl, pH 7.4, and centrifuged for 16 hr in a Beckman BIV rotor at 70,000*g* (3). The 40S and 60S fractions were collected and pelleted in a Beckman 60 Ti rotor for 8 hr at 240,000*g* (Fig. 1).

Agglutination tests. The agglutination tests were carried out with the antisera from goats, sheep, chickens, and rabbits which were injected (axilla) with the 60S ribosomal subunits in Freund's adjuvant (50%) with initial doses of approximately 1 mg each, 10 days apart and then followed by monthly doses of 1–5 mg (Antibodies, Inc.). Normal sera were obtained either from Antibodies,

Inc. or the Grand Island Biological Company.

The serum was prepared in serial 2-fold dilutions to 1:256 (14) by addition of 0.15 *M* NaCl and 0.1 ml of each dilution was added to tubes containing 0.1 ml of the cell suspension (10^7 cells/ml). The tubes were incubated in a 37° water bath for 1 hr and shaken every 20 min. At the end of the incubation, a drop of the suspension was examined microscopically. The percentage agglutination was determined from the proportion of cells in clusters to the total counted.

The types of cells used were Novikoff hepatoma ascites cells, the Walker 256 carcinosarcoma ascites cells and spleen white cells. The Novikoff hepatoma ascites cells were obtained from peritoneal fluid on the sixth or seventh day after transplantation. Approximately 8–10 days were required for the Walker tumor to produce an equivalent amount of ascites fluid. Tumor transplantation was carried out either in rats from the Holtzman Co. (Madison, Wis.) or Sprague-Dawley rats from the Schmidt Co. (Madison, Wis.).

After aspiration, the peritoneal fluid was centrifuged at room temperature for 3–4 min at 400*g*, and the pellet was dispersed in 0.15 *M* NaCl. This procedure was repeated several times until the final sediment contained very few red cells. Each milliliter of packed cells was resuspended in 10 ml of 0.15 *M* NaCl and the cell suspension used in the agglutination tests contained approximately 10^7 cells/ml.

Normal control white cells were obtained from the pooled spleens from at least six rats. The spleens were finely minced in a beaker containing 20 ml of 0.15 *M* NaCl, and the mince was then filtered through eight layers of gauze and centrifuged in the same manner as the tumor cells. For 0.1 ml of packed white cells, 2 ml of 0.15 *M* NaCl were added to provide a suspension with approximately 10^7 cells/ml.

Immunofluorescence. The Novikoff ascites tumor cell suspension containing 10^6 cells/0.1 ml was incubated with 0.1 ml of goat antiserum for 1 hr at 37°. The serum was then removed by washing the cells three times with 3 ml of 0.15 *M* NaCl containing 0.015 *M* potassium phosphate (pH 7.2). The washed cells were incubated with 0.1 ml of fluorescein-labeled rabbit antigoat γ-globulin at 37° for 1 hr. The labeled serum was removed by washing the cells three times with the same buffer. The washed cells were resuspended in a drop of buffered glycerol and examined with the fluorescence microscope.

Studies on tumor growth. In these experiments, the pelleted tumor cells were resuspended in NKM solution (containing 0.13 *M* NaCl, 0.005 *M* KCl, and 0.008 *M* $MgCl_2$) and recentrifuged. The supernatant was discarded and replaced with an amount of antiserum equivalent to the original amount of ascites fluid. The tumor cells plus antiserum were incubated together for 2 hr in a 37° water bath and shaken every 30 min. A normal goat serum control was included.

Following the incubation, the suspensions containing the cells and serum were centrifuged at 400*g* at room temperature for 3–4 min. The sedimented tumor cells were resuspended in sufficient NKM buffer to make the cell concentration 20×10^6 cells/ml and 2.5 ml were injected intraperitoneally into each rat. Tumor growth was evaluated on the seventh day after transplantation or at frequent intervals thereafter depending on the growth of the tumor.

Quantitative precipitin assay. The pelleted ribosomal subunits were suspended in 0.75 ml of 0.15 *M* NaCl, 0.015 *M* K_2HPO_4 (pH 7.2) at concentrations of 0.22–0.89 mg/ml and were incubated with 0.25 ml of antisera or normal sera for 2 hr at 37° and then kept for 18 hr at 4°. The precipitate was removed by centrifuging for 15 min at 1000*g* and washed twice in ice-cold 0.15 *M* NaCl. The protein content of the precipitate was determined by the method of Lowry *et al.* (15).

Method for γ-globulins. γ-Globulins were prepared from these antisera by the method of Kendall (16).

Results. Agglutination experiments. As indicated in Fig. 2 and Table I, 4+ agglutination occurred when the Novikoff hepatoma cells were incubated with the goat antisera to the tumor 60S ribosomal subunits. The rabbit antisera were negative and one chicken

and one sheep antiserum produced some degree of agglutination. Inasmuch as the titer of the antiserum from one goat (No. 195) was highest, all subsequent studies were carried out with this antiserum.

The Novikoff hepatoma ascites cells are

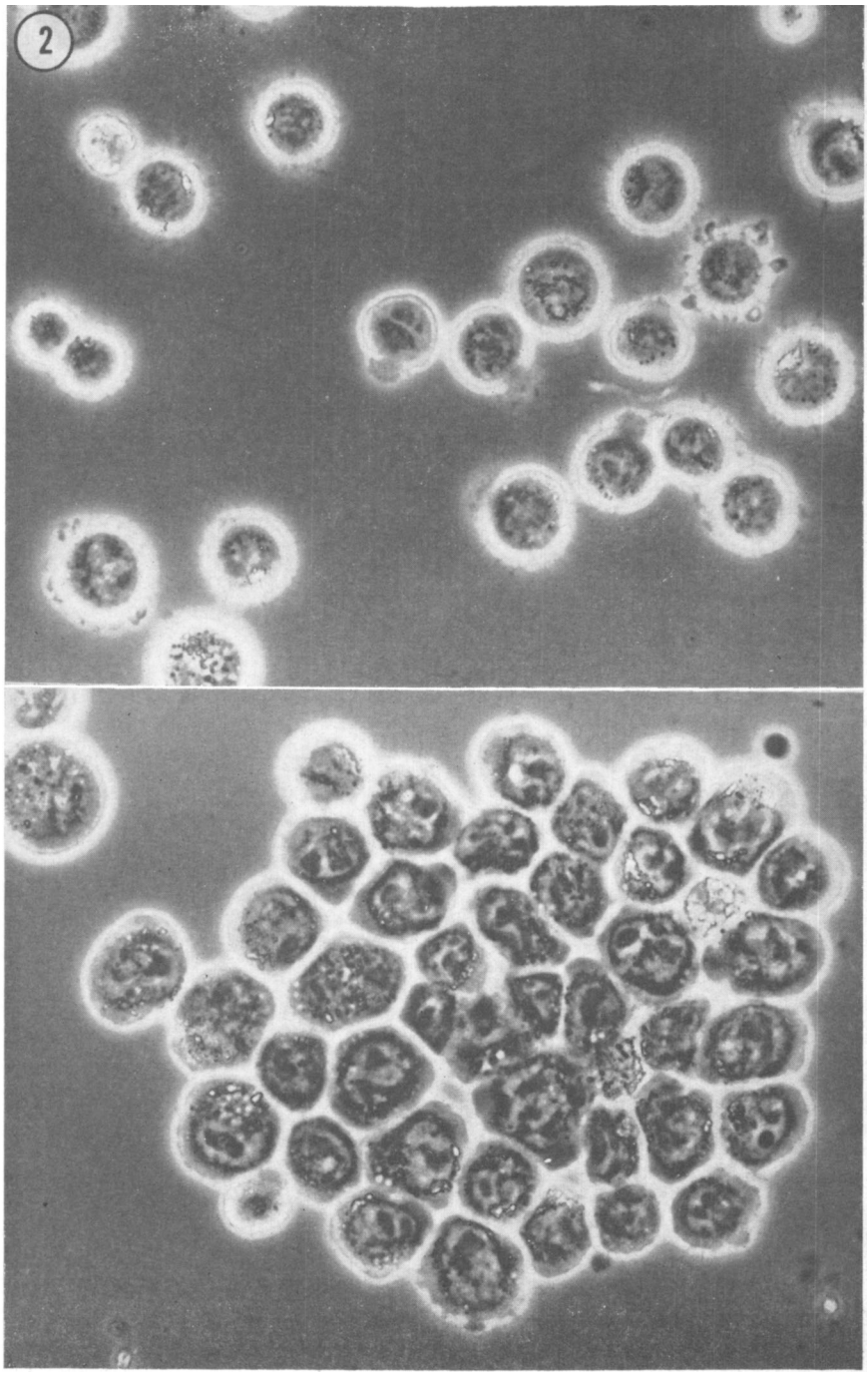

FIG. 2. Novikoff hepatoma ascites cells: (upper) cells incubated with normal goat serum; (lower) agglutinated cells after incubation with antiserum at 37° for 1 hr; ×1200.

TABLE I. Agglutinating Activity of Various Sera for Novikoff Hepatoma Ascites Cells.[a]

	Degree of agglutination
Antiserum	
Goat 195	++++
140	+++
Sheep 211	++
213	+
Rabbit 1-7	—
Chicken 14	++
9, 10, 11, 12, 13, 15	—
Normal Serum	
Goat	—
Sheep	—
Rabbit	—
Chicken	—

[a] In these experiments, the cells and undiluted sera were incubated for 1 hr in a 37° water bath. The extent of agglutination was determined by microscopic study of the cells. The experiments were carried out in duplicate in two separate experiments.

usually present as single cells in the peritoneal fluid as well as in 0.15 *M* NaCl. Figure 3 shows the percent agglutination of the Novikoff cells by the antiserum and the γ-globulin prepared from the antiserum. Both a commercially obtained goat antirat γ-globulin and normal goat serum did not produce significant agglutination of Novikoff hepatoma ascites cells.

Walker ascites tumor cells occur singly or in groups in the peritoneal fluid. Many of the cell groups are broken up during washing but some cells adhere to one another even when resuspended in 0.15 *M* NaCl. Although there was a "background" agglutination of 15% which occurred when the Walker ascites cells were incubated either with 0.15 *M* NaCl or normal goat serum (Fig. 4), there was significant agglutination of the Walker tumor cells with the antiserum to the Novikoff hepatoma 60S ribosomal subunits.

Spleen white cells had a background agglutination of 2%. With these cells the agglutinating effects were similar for both the normal goat sera and the antisera (Fig. 4).

Immunofluorescence. The Novikoff hepatoma ascites cells, which were incubated initially with the antiserum to tumor 60S ribosomal subunits followed by fluorescein-labeled rabbit antigoat γ-globulin, exhibited a ring-type fluorescence on their surfaces (Fig. 5). Following initial treatment of the cells with antisera to normal liver 60S ribosomal subunits, positive, but much weaker, intensities of fluorescence were observed. The cells treated initially with normal goat sera did not exhibit

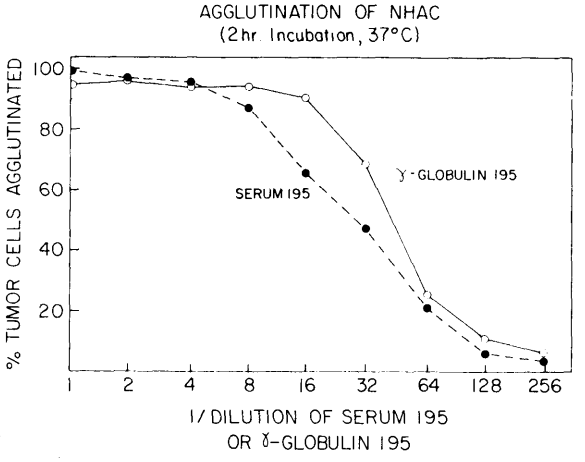

FIG. 3. Comparative agglutination of Novikoff hepatoma ascites cells by goat antiserum (No. 195 to tumor 60S ribosomal subunits and by γ-globulins from this antiserum. The conditions for these incubations are described in the text. Since the purified γ-globulin had only a slightly greater agglutinating activity than the serum from which it was derived, most of these experiments were carried out with the antiserum itself.

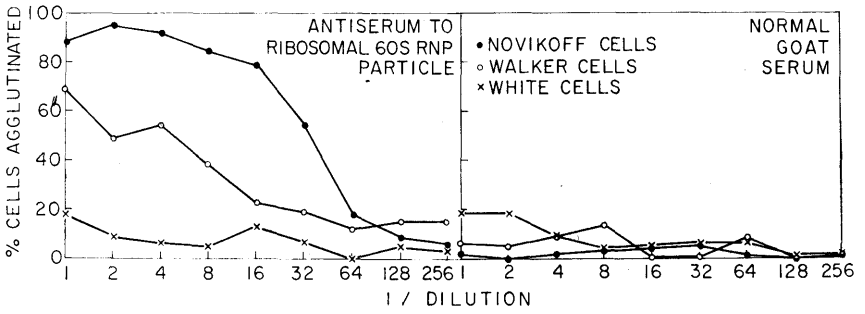

FIG. 4. Comparative agglutination of normal goat serum and the antiserum to the 60S ribosome subunit on Novikoff hepatoma cells, Walker carcinosarcoma ascites cells, and white cells derived from spleens. In these experiments the background agglutination of 15% was subtracted in the case of the Walker tumor cells.

significant fluorescence.

Suppression of tumor growth. Table II presents the results of experiments in which the Novikoff hepatoma ascites cells were incubated *in vitro* with the antiserum to the 60S tumor ribosomal subunits for 2 hr at 37° and then injected intraperitoneally into rats. Tumor growth was markedly decreased when the cells were incubated with antisera as compared to normal goat sera. On day 7, tumor growth was not evident in approximately half the animals that received an-

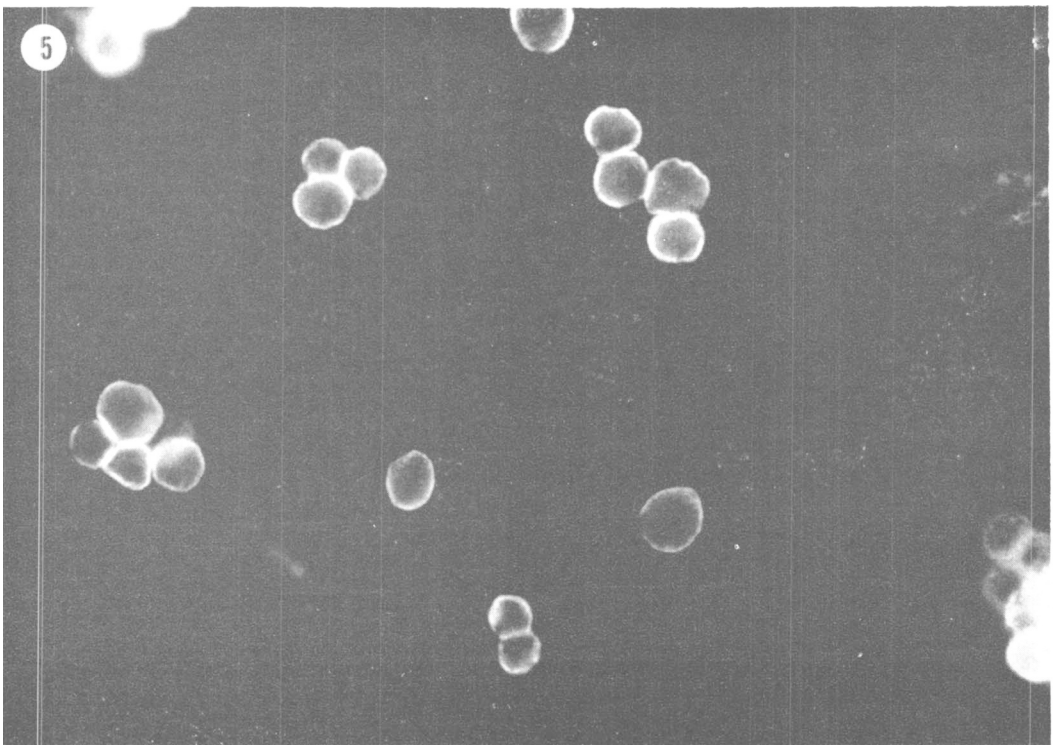

FIG. 5. Immunofluorescent stain of Novikoff hepatoma ascites cells. The Novikoff hepatoma ascites cells were incubated for 1 hr at 37° with the antiserum to 60S ribosomal subunits and then stained by an equal volume of fluorescein-labeled rabbit antigoat γ-globulin for 1 hr at 37°. Halos were seen around the periphery of these cells but none were observed when normal goat serum was initially incubated with these cells.

differ from the growth of CFU paralleling or perhaps preceding expansion of the myeloblast–promyelocyte compartment (17). From these observations, it has been suggested that the *in vitro* technique measures a granulocyte committed stem cell, a concept analogous to the erythroid committed stem cell (2) which is thought to be responsive to erythropoietin.

The material responsible for colony growth, colony stimulating factor (CSF), has been derived from a number of sources including kidney cells (10), embryonic tissue (18), fibroblasts (19), leukocytes (20), and certain sera (21) and urinary extracts (22). Although the diversity of sources might suggest that this material is a nonspecific growth factor, extensive supplementation of the tissue culture media with known nutrients does not lead to appreciable colony formation in the absence of CSF. Therefore, it would appear that CSF may represent a specific granulocyte stimulating factor.

At present, it is uncertain whether CSF may be a true granulopoietin despite its capability of inducing extensive proliferation and maturation of immature granulocyte precursors *in vitro*. Although it may be assumed that a granulopoietin would be released in response to granulocytopenia, only limited data is available regarding CSF levels with induced neutropenia. Studies in which CSF was determined at intervals after irradiation have led to conflicting results. Initial observations in mice indicated no appreciable change in serum CSF following 450 R of total body irradiation (5). More recent studies in which higher doses of irradiation were employed, showed heightened CSF at the nadir of neutropenia; levels of activity bore an inverse relationship to circulating granulocyte levels (6).

Our earlier studies indicated that acute neutrophil depletion in rats, using an antineutrophil serum, was associated with a marked increase in serum colony stimulating factor (9). The present studies extend and confirm these observations. However, cyclophosphamide-treated animals differ from those given antineutrophil serum in that peripheral granulocyte depletion is delayed, as is the CSF response. Moreover, the magnitude of response following administration of this alkylating agent does not approach that seen with acute granulocyte depletion. The latter difference may in theory be a manifestation of CSF turnover (9). Although utilization of CSF has not been demonstrated *in vivo*, consumption of this material appears to parallel the growth of developing granulocytic colonies *in vitro* (23).

The appearance of increased serum CSF after granulocytopenia induced by irradiation, or, as in the present experiments, after cyclophosphamide, suggests that CSF may be responsible for *in vivo* recovery of granulopoiesis. Further findings of a diffusable serum factor 3.5 to 4 days after irradiation, capable of stimulating the growth of myeloid cells implanted in Millipore chambers *in vivo* (24), suggest that certain humoral agents are involved in the recovery of granulopoiesis. The present observations suggest that CSF may be such a granulopoietin. However, it remains to be demonstrated that this factor is active *in vivo* and that it is responsible for control of normal granulopoiesis.

Summary. At intervals following a single injection of cyclophosphamide, groups of rats were tested for the presence of an *in vitro* granulocyte colony stimulating factor (CSF). Increased serum CSF activity was demonstrated on days 5 through 7 after injection, corresponding to a phase of marked peripheral granulocytopenia. The CSF returned to base line levels as peripheral blood granulocytes returned to normal. Increased elaboration of CSF in conjunction with granulocytopenia is in accord with a possible role of this material as a regulator of granulopoiesis.

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TABLE IV. Quantitative Analysis of Precipitated Antigen–Antibody Complexes of 40S Ribosomal Subunits and Antiserum.

Antigen		Protein in precipitate (μg)			
		Antitumor 60S ribosomal subunits		Antiliver 60S ribosomal subunits	
OD	(mg)	Liver 40S ribosomal subunits	Tumor 40S ribosomal subunits	Liver 40S ribosomal subunits	Tumor 40S ribosomal subunits
0.5	0.04	15 (10, 25)	10 (0, 20)	0	5 (0, 10)
1	0.09	35 (15, 50)	35 (15, 55)	20 (0, 40)	60 (50, 70)
2	0.17	50 (10–75)	115 (45–170)	70 (30–100)	130 (75–160)
4	0.33	130 (120–140)	185 (135–230)	170 (155–190)	150 (110–200)
6	0.50	145 (115, 180)	215 (160, 270)	225 (220, 225)	185 (140, 230)
8	0.66	170 (150, 190)	240 (160, 320)	170 (130, 245)	155 (110, 200)

[a] The conditions for these experiments are the same as those described in Table III.

was approximately one fourth of that with the 60S ribosomal subunits. Accordingly, there is some specificity of the precipitins for the 60S ribosomal subunits as compared to the 40S ribosomal subunits. In this case also, no significant differences were found for the tumor and the liver.

Discussion. Although a number of studies have shown that antibodies form following immunization of animals, particularly rabbits and horses with ribosomes and ribosomal products (6, 8–11), the present studies demonstrate the formation of agglutinins and precipitins following immunization of goats with 60S ribosomal subunits of tumor cells. The agglutinins are sufficiently effective to inhibit and in some cases to totally prevent growth of the tumors.

The original concept of this research stemmed from the possibility that antiribosomal antibodies might affect tumor cell surfaces if ribosomes were in close proximity to the plasma membrane as suggested by the studies of Warren *et al.* (13). Inasmuch as electron micrographs indicated that the 60S ribosomal subunits of the Novikoff hepatoma ascites cells did not contain membrane components, it seems unlikely that the agglutination resulted from formation of antibodies to plasma membrane components contaminating the 60S ribosomal subunits, although it is possible that either membrane precursors or micro quantities of membrane components may be present. On the other hand, it seems unlikely that such contaminants would be present in the tumor ribosomal subunit preparation and not in corresponding preparations in the liver. It is also possible that some of the components of the 60S ribosomal subunits of the tumor differ from those of the normal liver, and some of these may have antigenic determinants similar to those of surface antigens. In any event, the marked agglutination observed in these studies indicates that different antibodies are formed in response to immunization with tumor and liver 60S ribosomal subunits. On the other hand, no significant differences were detected between the precipitins resulting from immunization with normal liver or tumor 60S ribosomal subunits.

It is interesting that there was agglutination of the Walker tumor ascites cells with the antisera to the 60S ribosomal subunits of the Novikoff hepatoma ascites cells which suggests that the Walker tumor ascites cells contain antigenic determinants common to those of the Novikoff hepatoma ascites cells. On the other hand, the finding that spleen white cells were not affected by these agglutinins indicates that either they lack ribosomes adjacent to their surfaces or that their surface-associated ribosomes differ from those of the tumor cells.

The goals of further studies are both mechanistic and immunotherapeutic. From the mechanistic point of view it is of interest to define the types of agglutinins, their biochemical characteristics and the type of lesion they induce in the tumor cells. From the immunotherapeutic point of view it would be of considerable interest to determine

whether antibodies of considerably higher titer to tumor cells could be produced by specific ribosomal proteins or 28S ribosomal RNA itself, possibly coupled to "carriers" (7). It would be of interest if antisera could be developed to ribosomal subunits of human tumor cells such as leukemic cells with similar specificity for the tumor cells.

Summary. Goats immunized with 60S ribosomal subunits of Novikoff hepatoma ascites cells produced agglutinins for Novikoff hepatoma ascites cells and precipitins for 60S ribosomal subunits. These antibodies also agglutinated Walker 256 carcinosarcoma ascites cells but did not agglutinate spleen white cells. Goats immunized with 60S ribosomal subunits of normal liver did not produce agglutinins for either Novikoff hepatoma or Walker tumor cells but did produce precipitins for 60S ribosomal subunits. The precipitins did not show significant differences in their effects on tumor or liver 60S ribosomal subunits. However, on a molar basis, there was a greater precipitation of the 60S ribosomal subunits than the 40S ribosomal subunits. The antiserum to Novikoff hepatoma 60S ribosomal subunits markedly inhibited the growth of Novikoff hepatoma ascites cells.

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