COBRA VENOM-INDUCED HEMOLYSIS
ACTIVITY LEVELS IN SERA OF PATIENTS WITH NEOPLASTIC
AND OTHER DISEASES*

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Interest in the alternate or C3 shunt complement pathway has recently been
generated by a number of reports (1–5). Its presence in invertebrates points to a
primitive phylogenetic appearance (6). The C3 shunt can be triggered by high molec-
ular weight polysaccharides in a manner reminiscent of the properdin system (7).
Once activated by cobra venom, erythrocytes and other cells can be lysed in the ab-
sence of specific antibody (3). This mechanism may also be set into motion by im-
munoglobulins to which the classical C sequence is indifferent (2–4). Moreover,
cleavage of C3 via the alternate pathway results in the production of biologically active
substances (3, 8).

To facilitate further studies of the C3 shunt system, a hemolytic assay has
been described (3, 5). It has now been used to measure the C3 shunt hemolytic
activity in the sera of patients with neoplastic and other diseases. The data
suggest that human neoplasia may be associated with changes in the C3 shunt
complement system, particularly as it concerns one of its major components,
the C3 proactivator (3).

Materials and Methods
Sera. Sera from patients with a variety of malignant tumors (colon and breast carcinoma,
sarcoma, leukemia, etc.) were obtained through the generous cooperation of Doctors Lloyd Old
and Germain Trempe of the Sloan-Kettering Institute for Cancer Research in New York. Sera
from healthy adults were obtained from laboratory personnel and donors at the blood bank of
the New York University Medical Center. Sera from hospitalized patients with diseases other
than cancer (hypertension, cardiac disease, cirrhosis, thrombotic phenomena, etc.) were made
available through the kind assistance of Dr. Gerald Salen of the Veterans Administration

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Hospital in Manhattan and Dr. Franco Cavallo of Bay Shore, Long Island, N.Y. All the specimens were stored at −70°C until used.

**CVFAH<sub>50</sub> Assay.**—This assay was performed as in reference 5. Briefly, cobra venom factor (CVF; Cordis Laboratories, Miami, Fla.) was incubated with several dilutions of the test serum in Mg<sup>2+</sup>-Veronal-buffered saline (VBS) before the addition of guinea pig C and a suspension of guinea pig erythrocytes, both rendered 0.02 M in respect to ethylenediaminetetraacetate (EDTA). After further incubation the degree of lysis was measured spectrophotometrically. The term CVFAH<sub>50</sub> (CVF activable hemolysis) units per milliliter refers to the reciprocal of the serum dilution required to lyse 50% of the guinea pig red cells (5).

**Inulin Treatment.**—0.5 ml of undiluted serum was incubated with 0.5 ml of inulin suspension (5 mg) in Mg<sup>2+</sup>-VBS for 30 min at 37°C. The tubes were centrifuged and the supernates assayed for CVFAH<sub>50</sub>.

**TABLE I**

| Clinical status | Units/ml serum |
|-----------------|----------------|
|                 | Total | 10-20 | 20-30 | 30-40 | 40-50 | 50-60 | 60-70 | 70-80 | >80 |
| A) Healthy      | Number | 81    | 5     | 30    | 20    | 12    | 7     | 4     | 2    | 1   |
| B) Hospitalized | Number | 50    | 6     | 19    | 12    | 9     | 3     | 1     | 0    | 0   |
| Noncancer       | 131   | 100   | 8.4   | 37.4  | 24.4  | 16.0  | 7.6   | 3.8   | 1.5  | 0.8 |
| C) Hospitalized | Number | 87    | 0     | 14    | 19    | 21    | 13    | 11    | 5    | 4   |
| Cancer          | Per cent | 100  | 0     | 16.1  | 21.8  | 24.1  | 14.9  | 12.6  | 5.7  | 4.6 |

Mean ± sd: group A, 35.6 ± 14.5; group B, 32.3 ± 11.5; group C, 47.7 ± 18.2.

**Immunoelectrophoresis.**—This procedure was carried out with 1.0% agarose in Veronal buffer (pH = 8.6, μ = 0.05) in 0.01 M EDTA for 120 min at room temperature with a potential gradient of 5 V/cm. The lines of precipitation were developed with monospecific goat antihuman C3 (Cordis Laboratories) and with rabbit anti-human β2 glycoprotein II (Behring Diagnostics, Somerville, N. J.).

**RESULTS AND DISCUSSION**

The data in Table I show that the mean CVFAH<sub>50</sub> titers for the healthy donors, group A, and for the patients with non-neoplastic diseases, group B, were similar, i.e., 35.6 and 32.3 CVFAH<sub>50</sub> units/ml, respectively (t = 1.44, P > 0.1). Nor did the distribution frequencies of the titers in these two populations differ significantly (χ² = 2.1, P > 0.8).

In contrast, the mean serum titer for the cancer group (group C), 47.7, was significantly higher than that for group A (t = 4.76, P < 0.001), group B (t = 6.02, P < 0.001), and for the combined value of group A plus group B (t = 5.81, P < 0.001). Likewise, the distribution profile of the serum titers for the cancer group differed from that of the other two groups, C vs. A + B; (χ² = 24.56, P < 0.001) (Fig. 1 A).
The contribution of the cancer and noncancer sera to each titer interval was expressed as the per cent of the total number of sera within that class, Fig. 2 A. The sera of the cancer patients contributed most of the higher titer values.

Fig. 1. Distribution of CVFAH_{26} titers in sera of cancer and noncancer donors before and after inulin treatment.

Fig. 2. Per cent contribution of cancer and noncancer sera to each titer class before and after inulin treatment.

However a considerable overlap between the two sets of data is apparent (Figs. 1 A and 2 A).

The inference that sera from cancer patients may differ from those in the control groups in respect to the C3 shunt system was strengthened by other experiments. Since the interaction of fresh serum with inulin results in marked
alteration of the C3 proactivator (3, 5) we examined the effect of this polysaccharide on CVFAH₉₀ titers. Inulin treatment reduced the mean titer of the sera in groups A and B to the same extent (t = 0.67, P > 0.6). These data have therefore been pooled for the comparisons shown in Table II and Figs. 1 B and 2 B. Inulin treatment heightened the differences between the cancer and control groups in respect to mean titer values (t = 6.69, P < 0.001), distribution frequencies (χ² = 28.78, P < 0.0005), and hemolytic activity losses (Figs. 1 B and 2 B, and Tables II and III).

Differences between the cancer and noncancer sera were also demonstrable

| Clinical status | Total no. | <10 | 10-20 | 20-30 | 30-40 | 40-50 | >50 |
|----------------|-----------|-----|-------|-------|-------|-------|-----|
| Healthy and noncancer | 64 | 5 | 36 | 20 | 2 | 1 | 0 |
| Cancer | 43 | 0 | 6 | 17 | 9 | 6 | 5 |

The means ± sd for the control and cancer group were 18.0 ± 7.7 and 31.3 ± 11.3 respectively; t = 6.69, P < 0.001.

| Clinical status | Total no. | 0-20 | 20-30 | 30-40 | 40-50 | >50 |
|----------------|-----------|------|-------|-------|-------|-----|
| Noncancer | 64 | 0 | 4 | 15 | 36 | 19 |
| Cancer | 43 | 11 | 13 | 12 | 2 | 5 |

χ² = 36.52, P < 0.0005.

The mean losses in activity due to inulin treatment of the noncancer and cancer sera were 46.4 ± 12.2 and 29.4 ± 12.8% respectively; t = 6.82, P < 0.001.

by immunoelectrophoresis as exemplified in Fig. 3. Heavier precipitates generally appeared with the cancer sera after development with an antiserum to human β2 glycoprotein II. After inulin treatment the line of precipitation formed by the control sera, in the β region, was largely converted to one of γ mobility. Here, too, the cancer sera behaved differently in that conversion as a consequence of inulin treatment was less complete (Fig. 3). The differences in the intensity of the precipitates and the extent of conversion generally agreed with the CVFAH₉₀ titers. C3 was partially cleaved in all sera after inulin treatment (Fig. 3).

22 sera in the control groups and 17 cancer sera were assayed concurrently for CVFAH₉₀ and hemolytic C titers, CH₉₀. The latter were performed as in reference 2 with sensitized sheep erythrocytes. No significant difference in mean...
CH₄₀ titers was observed between the two groups of sera \((t = 1.2, P > 0.2)\). The correlation coefficients for the two assays in the cancer and control groups were \(r = 0.22\) and 0.44, respectively.

The present findings indicate that, as a group, the sera of cancer patients exhibit a different C3 shunt reaction pattern than do the sera of healthy donors or those with non-neoplastic diseases. The cancer sera generally manifest greater hemolytic activity after admixture with a cobra venom protein, the terminal complement components (C-EDTA), and unsensitized guinea pig erythrocytes. In addition, they retain relatively more hemolytic activity than do the controls after the incubation with inulin (Figs. 1 and 2). The higher titers of the untreated and inulin-treated cancer sera may reflect increased levels of C3 pro-

![Fig. 3. Immunoelectrophoresis of cancer sera (c) and controls (nc). The results obtained with six sera are shown before (con, upper three wells) and after treatment with inulin (inu, lower three wells). The troughs contained anti-human β2 glycoprotein (α-bg) or anti-human C3 (α-c3). The numbers beside the designations c or nc refer to the CVFAH₄₀ titers for these specimens.](image)

activator as judged by immunoelectrophoresis (Fig. 3). This interpretation, consistent as it is with the present data, does not exclude the possibility that other factors \((9, 10)\) may account in part for the observed differences. Further characterization of the reaction steps in this sequence coupled with detailed clinical studies are required to evaluate the implications of these findings.

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REFERENCES

1. Gewurz, H., H. S. Shin, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxin lipopolysaccharides: consumption of each of the six terminal complement components. J. Exp. Med. 128:1049.
2. Osler, A. G., B. Oliveira, H. S. Shin, and A. L. Sandberg. 1969. The fixation of guinea pig complement by γ1 and γ2 immunoglobulins. J. Immunol. 102:269.
3. Götze, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternate pathway of complement activation. J. Exp. Med. 134:908.
4. Sandberg, A. L., and A. G. Osler. 1971. Dual pathways of complement interaction with guinea pig immunoglobulins. J. Immunol. 107:1268.
5. Brai, M., and A. G. Osler. 1972. Studies of the C3 shunt activation in cobra venom induced lysis of unsensitized erythrocytes. *Proc. Soc. Exp. Biol. Med.* 140:1116.

6. Anderson, R. S., N. K. B. Day, and R. A. Good. 1972. Specific hemagglutinin and a modulator of complement in cockroach hemolymph. *Infect. Immun.* 5:55.

7. Pillemer, L., L. Blum, I. H. Lepow, E. W. Todd, and A. C. Wardlaw. 1954. The properdin system and immunity. *Science (Wash. D.C.)*. 120:279.

8. Oliveira, B., A. G. Osler, R. P. Siraganian, and A. L. Sandberg. 1970. The biological activities of guinea pig antibodies. *J. Immunol.* 104:320.

9. Alper, C. A., I. Goodkofsky, and I. H. Lepow. 1972. Studies of glycine-rich β glycoprotein (GBG), properdin factor B and C3 proactivator (C3PA). *Fed. Proc.* 31:787. (Abstr.)

10. Hunsicker, L. G., S. Ruddy, and K. F. Austen. 1972. Additional factors required for cobra venom induced activation of C3. *Fed. Proc.* 31:788. (Abstr.)