Antibody-Mediated Suppression of Grafted Lymphoma Cells. I. Participation of a Host Factor(s) other than Complement (36216)

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Humoral antibodies directed against certain transplanted lymphomas and sarcomas of mice may suppress grafts of these tumors in syngenic and allogenic recipients (1–4). Studies *in vitro* and *in vivo* have suggested the participation of serum complement (1, 2) and macrophages (4, 5) in suppression. Experiments *in vitro* also have indicated that antibody alone (6) or in association with lymphocytes (6–8) may be an effector. The present studies were undertaken to ascertain the relative significance of these humoral and cellular factors in inhibiting the growth of *syngeneic* lymphoma transplants in mice when alloantibody directed against and admixed with the tumor was administered to the graft recipient.

Materials and Methods. Mice. Inbred mice of strains C3HeB, C57BL/6 (hereafter abbreviated B6), and B10.D2-new, with complete complement systems, and AKR and B10.D2-old, deficient for component C5 (9–11), were purchased from The Jackson Laboratory, Bar Harbor, ME. The mice were 8–12 weeks old on introduction to the experiments.

Tumors. The C3H lymphoma 6C3HED, dating from 1941, was obtained from The Jackson Laboratory; it is maintained by subcutaneous passage in C3HeB females. Lymphoma L707 arose in the thymus of an X-irradiated B10.D2-old female in 1970 and is maintained by sc passage in B10.D2-old females. It grows progressively in B10.D2-new mice, but somewhat more slowly than in the "old." It was in its 12th transplant generation at its use in the experiments.

Experimental tumor grafting. Tumor donors were killed by asphyxiation with CO_2. The tumors were removed, moistened with Hanks' balanced salt solution (BSS), and minced with scissors. The mince was pressed through a stainless-steel screen into BSS and the recovered cells were washed three times by centrifugation in BSS at room temperature. After the last wash several minutes were allowed for cell clumps to settle in the centrifuge tube. The top two-thirds of the supernatant was then aspirated and its cell count was adjusted to 4×10^6/ml BSS. (Previous experience justifies an estimate of more than 90% viability for these cells.) All procedures were carried out under aseptic conditions with sterile instruments and equipment.

For injection, the cell suspensions were mixed with equal volumes of either BSS or alloantibody just before they were inoculated. Each mouse received 0.05 ml of the suspension in one calf muscle (*i.e.*, 10^5 tumor cells). Growth of the grafts was followed by caliper measurements of calf size in two diameters taken at right angles to each other. Values are recorded as the average of the sum of the two diameters for each measurement.

In some experiments, the cells were injected intraperitoneally. For this, nine parts of BSS were mixed with 1 part of the tumor–BSS or tumor–antibody mixture and 0.5 ml of this suspension (*i.e.*, 10^5 cells) was injected. Survival times of the hosts were recorded.

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Antibodies. B6 anti-6C3HED was raised in B6 females given intraperitoneal injections of freeze-dried 6C3HED (10 mg dry weight per injection in 0.2 ml of 0.15 *M* NaCl). The initial injection was emulsified in an equal volume of Freund's complete adjuvant. Four injections were given at weekly intervals. The mice were bled from an incision of the ventral tail artery 7 days after the last injection. Booster injections (for a total of four boosters) were given 10–20 days after each bleeding and the animals were bled 1 week later. Serum was collected after the blood had clotted at room temperature and had an overnight residence at 4°.

AKR anti-L707 antibody was obtained in peritoneal ascites fluid which developed in AKR females in response to ip injection of Freund's complete adjuvant (12). Primary immunization was with a homogenate of live B10.D2-old spleen, thymus, submaxillary salivary glands, and L707 in BSS, one donor per five recipients per injection. Six injections of 0.2 ml of homogenate were given ip twice weekly for 3 weeks. The mice were tapped for ascites fluid at 9 and 14 days after the last injection. Adjuvant was given in three injections of 0.2 ml each at 14, 11, and 9 days before the first tapping. Booster injections of L707 (0.2 ml ip) were given at 2 and 4 months after the last primary injection, and the mice were tapped at 7, 10, and 14 days after each booster. Production of ascites fluid was maintained by a single ip injection of 0.2 ml adjuvant at the time of each booster injection. Antibody activity was attested by hemagglutination and by cytotoxicity *in vitro* for appropriate target cells.

Rabbit anti-guinea pig C3 was prepared as previously described (13).

Cobra venom factor. An anticomplementary factor from cobra (*Naje haje*) venom was purified by a method described previously (14).

Immunoelectrophoresis. The presence of C3 in mouse serum was determined by Scheidegger's method (15), with 0.5% agarose (Mann Research Laboratories, New York City) as the supporting matrix. After electrophoresis, rabbit anti-guinea pig C3 was added to the troughs and precipitin lines were given overnight to develop.

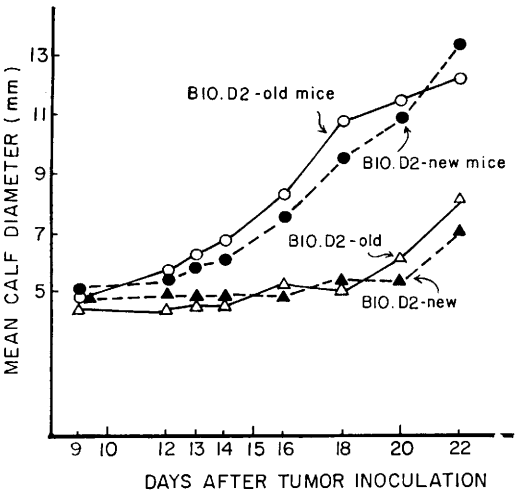

FIG. 1. Growth of lymphoma L707 in the calf muscle of B10.D2-old and B10.D2-new mice (5 females per group). ▲--▲, △—△ grafts of tumor cells mixed with AKR anti-L707 serum (raised in ascites fluid) before inoculation; ●--●, ○—○ tumor cells mixed with "normal" AKR ascites fluid before inoculation.

Irradiation. Whole-body and local X-irradiation was done with a GE Maxitron at a rate of 70 R/min, 250 kV, 20 mA, target distance 56 cm, with filters of 0.5 mm Cu and 1.0 mm Al. The mice were on a platform rotating at 7 rpm. They were confined in a plexiglas container for whole-body exposure. For local irradiation, the mice were anesthetized with sodium pentobarbital and were immobilized with masking tape on the platform. Shielding was with lead sheets 3 mm thick.

Complement titration. Mouse C3 was titered by the use of guinea pig complement components as described in (16). Whole complement, C1, and C-EDTA were assayed according to (17), C4 by the method of Hoffman *et al.* (18), and C2 as described in (19). Assays of whole complement were done the day the animals were bled and before the serums were frozen. Activity of serum from irradiated mice was expressed as percent of normal controls bled the same day. Measurements of other complement activities were made on serums stored at —70° and their activities were expressed as percent lysis.

Results. Suppression of L707. AKR anti-

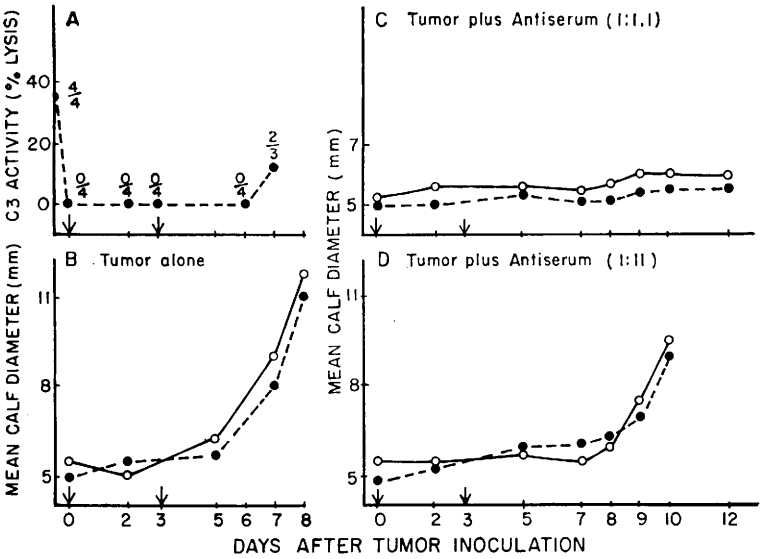

FIG. 2. Growth of lymphoma 6C3HED in calf muscle of normal C3HeB mice or C3HeB mice decomplemented by cobra venom factor (4 females per group). Arrows indicate days venom factor was injected ip. Mean calf (tumor) diameters in normal (O—O) and the decomplemented mice (●- - -●) given: A, Hemolytic C3 levels in venom-treated mice at 1:100 serum dilution; fractions are number of mice with immunoelectrophoretically detectable C3 per total animals. B, tumor alone; C, tumor mixed with antiserum diluted 1:1.1; D, tumor mixed with antiserum diluted 1:11. Antiserum given to decomplemented mice was decomplemented by incubation with venom factor (2 μg/ml serum) before mixing with tumor.

L707 suppresed grafts of the lymphoma (indigenous to B10.2D2-old mice) to the same extent in B10.D2-old and B10.D2-new mice (Fig. 1). The antiserum lacks C5 (for which AKR and B10.D2-old mice are deficient). Obviously, the presence of C5 is not essential for the suppressive action of the antiserum.

Effect of decomplementation of the host on the suppressive action of B6 anti-6C3HED. Whether an intact complement system is essential for tumor suppression was assessed for grafts of lymphoma 6C3HED (indigenous to the C3H strain) in C3H mice. This strain's complement system is intact. The future graft recipients were decomplemented with cobra venom factor. At a dose of 10 μg of the factor given ip twice at 3-day intervals, C3 activity disappeared and was undetectable for 6 days after the first injection (Fig. 2). There was no C3 hemolytic activity and C3 was not demonstrable by immunoelectrophoresis. Despite the absence of C3, B6 anti-6C3HED at two dose levels was fully suppressive when the tumor cell–antiserum mixtures were inoculated in a calf muscle 6 hr or 1 day after the first injection of venom factor (Fig. 2). A second experiment with three male and three female C3HeB recipients per experimental and control groups gave similar results. In another trial tumor alone was inoculated ip in three males each per decomplemented and control groups. The decomplemented mice died 13 days later; one control died on day 13 and two on day 14. When antibody (diluted 1:4) was mixed with the tumor inoculum, the three decomplemented males died on day 19; two of the controls died on day 19 and one on day 20.

Effect of whole-body irradiation. One day after irradiation with either 400 or 500 R, C3HeB mice received an inoculum of 6C3HED cells, with or without admixed antiserum, in one calf muscle. The serum did not suppress tumor growth in the irradiated mice (Fig. 3). Apparently, a radiosensitive host factor(s) cooperates with antibody in suppression. The grafts grew more quickly in the irradiated mice than in the controls. There were no significant

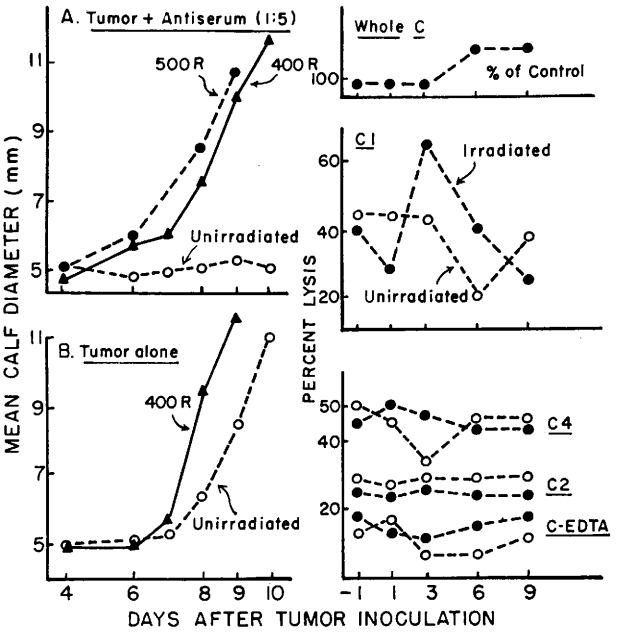

FIG. 3. Effect of whole-body irradiation on antibody-mediated suppression of lymphoma 6C3HED and on complement activity in C3HeB mice: A, Growth of tumor mixed with antiserum (diluted 1:5) in calf muscle of males (5 per group) given 500 R X-rays (●- - -●); and in 4 females per group either nonirradiated (○- - -○) or given 400 R (▲—▲); B, Tumor growth in nonirradiated or irradiated females (4 per group) given tumor alone.

Complement activity in males given 500 R (●- - -●) or nonirradiated (○- - -○) (6 mice per group) shown in right graphs. Whole C activity expressed as percent CH_{50} units for X-rayed mice against normal control values. C1, C4, C2, and C-EDTA assayed at serum dilutions of 10^{-5}, 10^{-2}, 10^{-2}, and 1:5, respectively; component activities expressed as percent lysis.

decreases in C1, C4, C2, and C-EDTA levels during 9 days after irradiation. Increases in whole complement levels were found in irradiated mice on days 6 and 9.

Effect of local irradiation. In an attempt to delineate the radiosensitive host factor, C3HeB mice with all of the body except the leg shielded by lead were given 400 R X-rays. Irradiated controls were not shielded. A second control group was not irradiated. 6C3HED cells admixed with antiserum were inoculated in the left calf muscle. Tumor growth was suppressed equally in the nonirradiated controls and the mice irradiated only in the leg. There was no suppression in the unshielded irradiated mice.

Discussion. The complement system has numerous biological functions which may be relevant to the immune destruction of tumor cells. Thus, sensitized target cells can be lysed *in vitro* by the intact complement system. Cells carrying complement components up to C8 can be lysed by normal lymphocytes without added C9 (20). Under some conditions C3 through C9 can be activated without the involvement of C4 or C2 (21–23). As yet no mechanisms have been found, however, for bypassing C3 and C5 for the lytic reaction. In addition to lysis, factors which promote immune adherence and phagocytosis, and increase vascular permeability and cause the accumulation of polymorphonuclear and mononuclear leukocytes, are released from C3 and C5 on activation of the complement system (24–26).

Though it has been suspected that complement may participate in antibody-mediated destruction of tumor cells, its role has not been defined in the mouse. 6C3HED cells have been lysed *in vitro* on exposure to alloantibody and guinea pig complement; mouse complement was ineffective (1). Enhanced destruction of grafts of this tumor occurred in mice given

guinea pig complement (1, 2).

Grafts of the strain A tumor, Sarcoma I, were found to be more effectively suppressed in preimmunized B10.D2-new than in B10.D2-old (deficient for C5) mice (27). The precise role of C5 cannot be inferred from this experiment since its absence may have influenced either the response to active immunization or destruction of the tumor. It is also possible that the B10.D2-new-Sarcoma I combination exhibited non-H-2 transplantation differences which are not present in the B10.D2-old combination. (Though the two strains of mice presumably should be coisogenic, skin grafts from B10.D2-new to B10.D2-old are rejected; they are accepted in the reverse direction [(28) and our unpublished data].)

Our results indicate that C3 and C5 are not required for antibody-mediated destruction of our test lymphomas. The situation, of course, may be different for other tumors and for species other than mice. Whether other complement components are essential has not been examined. Whether or not their presence is a requisite for antibody-mediated tumor suppression, our results with irradiation betoken an as yet undefined radiosensitive host factor(s) as an essential participant. The nature of this factor is under study.

It is of note that our experiments deal with so-called syngeneic grafts and with alloantiserums directed against the grafts. The grafts must share transplantation antigens in common with their hosts. The suppressive activity we find most probably is directed against antigenic specificities peculiar to the tumors. The antigens may be associated with an assumed viral etiology of the lymphomas but in view of the long transplantation history of 6C3HED this tumor may have acquired additional novel specificities (which are not to be considered "tumor specific") as a consequence of genetic mutation.

Summary. Humoral alloantibodies directed against two transplanted lymphomas suppressed the growth of these tumors as effectively in syngeneic mice severely depleted of C3 or genetically deficient in C5 as in controls with normal complement activity. In addition, whole-body irradiation at 400 R or higher abolished the animal's ability to suppress the tumor grafts in the presence of antibody even though irradiation did not diminish complement activity. It is concluded that the complete complement system is not needed for the antibody-mediated suppression of the lymphomas. An as yet unidentified host factor(s) distinct from the complement system is needed for this suppressive reaction.

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