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11. Spectrophotometric assays of NADPH oxidase were carried out at 37°C in cuvettes with a 1-cm light path, in a final volume of 1 ml; 0.1*M* phosphate buffer (*p*H 7.0) and a final concentration of 1 m*M* KCN; 0.1 m*M* NADPH; granule enzyme, 1 mg of protein.
12. Oxygen electrode assays of NADPH oxidase were carried out with a Clark membrane electrode (Instrumentation Laboratory, Inc., Boston) and a circulating water bath electrode chamber. The final volume was 1.3 ml of 0.1*M* phosphate buffer, *p*H 7.0 at 37°C. Final concentrations of KCN were 1 m*M*; 7 m*M* NADPH; granule enzyme, 2 mg of protein.
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6 September 1968

Human Osteosarcomas: Immunologic Evidence Suggesting an Associated Infectious Agent

Abstract. *Immunofluorescent studies have revealed a high incidence of antibodies to osteosarcomas in the serums of patients with this disease and their close associates which react with a common antigen (or antigens) in osteosarcomas. The distribution of these antibodies suggests the association of an infectious agent with this neoplasm which is capable of producing unrecognized infections in healthy contacts of these patients.*

Although viruses have been established as oncogenic agents capable of inducing a wide variety of animal neoplasms, their role in the etiology of human neoplasia remains undetermined. This report will summarize studies on the distribution of antibodies to osteosarcoma in patients with osteosarcoma, their immediate families, and their close associates which suggests the association of an infectious agent with this neoplasm.

Serums randomly selected from four patients with osteogenic sarcoma, 14 members of their immediate families, four close associates of one patient, and 25 normal blood-bank donors have been tested by the indirect immunofluorescence technique for antibodies against four different osteosarcomas. Imprints of osteogenic sarcoma tissue were made on glass slides and stored in a liquid-nitrogen refrigerator until used in the immunofluorescence tests with techniques that have been reported (*1*).

When the previously frozen tumor imprints were used in this test they were fixed in acetone for 10 minutes and allowed to dry; then a drop of test serum was added. The slide was incubated at room temperature for 20 minutes in a moist chamber. Smears were washed twice in saline and a drop of properly diluted horse antiserum to human globulin, conjugated to fluorescein isothiocyanate and previously absorbed with porcine liver powder, was added for another 20 minutes. The smears were again washed twice, mounted in 50 percent glycerin in saline, and examined with a fluorescent microscope. Serums were routinely tested at dilutions of 1:10, 1:100, and 1:300 and only at the higher dilutions if they were positive at 1:300.

The direct immunofluorescent technique was used to study the reactivity of globulins from two patients against their own osteosarcomas (patients B and C). Globulins were precipitated from serum with ammonium sulfate and conjugated to fluorescein isothiocyanate. The labeled globulins were then tested for their reactivity against osteosarcomas by allowing a drop of these conjugated antiserums to incubate on the tumor imprint slide for 30 minutes, after which the slide was washed twice and examined. Blocking studies were performed by incubating the smears with unlabeled serum, washing, and then adding the labeled globulin. This was allowed to incubate for 30 minutes, after which the slide was washed twice and examined.

Results of this study are summarized in Tables 1 and 2. Each patient's serum was found to react not only with his own osteosarcoma but also with the tumors of other patients (Table 1). The titer of reactivity varied between 1:100 and 1:600 (Table 2). Some serums (particularly those of high titer) showed prominent prozones that were negative when tested undiluted or at a 1:10 dilution, but that became strongly positive at dilutions of 1:100 or 1:300. The immunofluorescence staining was principally limited to the cytoplasm and cell membrane, but faint nuclear staining was also observed (Fig. 1). The specificity of these immunofluorescence reactions was studied by testing high-titer serum from the four osteosarcoma patients at dilutions of 1:10, 1:100, and 1:300 against human neoplasms of other types (a melanoma and adenocarcinomas of the breast and colon). No reactions were observed between serums from osteosarcoma patients and these three neoplasms.

A high incidence of immunofluorescence antibodies was also demonstrated in serums from members of the immediate family (85 percent) and the serums from close associates of patient B (91 percent). The tests of serums from normal blood-bank donors revealed a significantly lower

Table 1. Incidence of antibodies against four different osteosarcomas in the serums of patients, their relatives, close associates, and normal blood-bank donors. Results are expressed as No. positive/No. tested.

Type of serums	Osteosarcoma from patient A	Osteosarcoma from patient B	Osteosarcoma from patient C	Osteosarcoma from patient D	Total	Percent positive
Osteosarcoma patients	2/2	4/4	3/3	4/4	13/13*	100
Patients' family members	5/11	7/7	8/8	14/14	34/40*	85
Associates of patients	1/1	4/4	3/3	3/4	11/12*	91
Blood-bank donors	1/10	4/19	8/25	7/16	20/70	29

* Incidence of reactivity is significantly different from blood-bank donors ($P < .01$).

Table 2. Distribution of titers of antibodies to osteosarcoma in the serums of osteosarcoma patients, their relatives, close associates, and normal blood-bank donors.

Type of serums	No. of tests*	Distribution of antibody titers					Total positive (No.)	Percent positive †
		< 1:10	1:10	1:100	1:300	1:600		
Osteosarcoma patients	9			4	2	3	9	100
Patients' family members	21			14	6	1	21	100
Patients' associates	9	1		4	2	2	8	89
Blood-bank donors	48	36	3	7	2		12	25

* Data derived from testing of the same serum against osteosarcomas B, C, and D (see text). † Serums with titers of 1:10 or greater are regarded as positive.

incidence of this antibody (29 percent) than in the serums of patients, their relatives, or close associates. However, the number of serums tested against each osteosarcoma varied, depending upon the number of serums and tumor imprints of a particular type available for study at the time the tests were performed (Table 1). Since every serum was not tested against every osteosarcoma, it was possible that the random selection of serums on this basis had created a bias. To exclude this possibility, the data were recalculated, considering only the results of testing samples of the same group of serums against the same osteosarcomas. Table 2 gives the results of testing dilutions of the same serums from three osteosarcoma patients, seven family members, three close associates, and 16 blood-bank donors against imprints of the same three osteosarcomas. [Data from serums tested against osteosarcoma from patient A (Table 1) were excluded because so few serums from patients, patients' associates, and blood-bank donors were tested against this osteosarcoma.] The titers as well as the incidence of antibodies to osteosarcoma in serums from patients, patients' family members, and patients' associates were again significantly higher than those in normal serums (Table 2).

Direct immunofluorescent technique, with the use of labeled globulins from patients B and C against the four osteosarcomas, revealed immunofluorescent staining qualitatively similar to that observed by the indirect technique. The specificity of this reaction was evaluated by blocking studies. By this technique unlabeled serums from all four osteosarcoma patients were tested for their ability to block the reaction of fluorescein-labeled globulins from patients B and C against their own osteosarcoma cells. All serums tested from osteosarcoma patients were capable of blocking this reaction, indicating that all of these serums contained antibodies against the same or a closely related osteosarcoma antigen. The possibility of isoantibodies being responsible for these observations is excluded by the reactivity of each patient's serum with his own osteosarcoma and the blocking of this reaction by the reactive serums of others.

The increased incidence and higher titers of antibodies to osteosarcoma in serums from relatives and close associates of osteosarcoma patients suggests the association of an infectious agent with this neoplasm. The agent appears to be capable of infecting close associates of the patients, in whom it elicits the formation of antibodies reactive with an antigen (or antigens) in osteosarcomas. The complete cross-reactivity between serums from the family members of one patient with another patient's osteosarcoma suggests that these agents are antigenically similar if not identical.

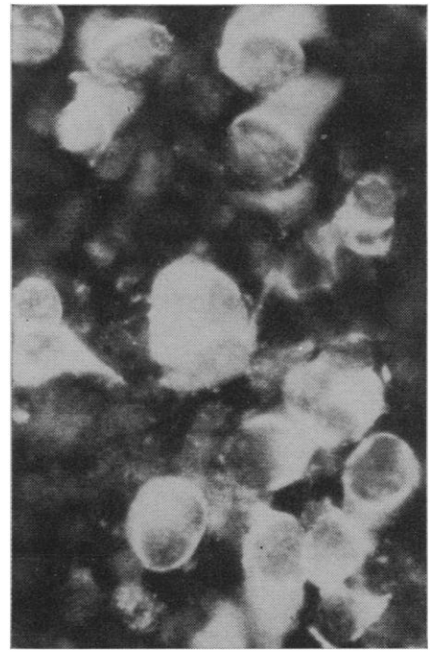

Fig. 1. Immunofluorescent photomicrograph of patient B's osteosarcoma imprint. Section was incubated with autologous serum, washed, and stained with fluorescent horse antiserum to human globulin. Note prominent cytoplasmic and weak nuclear fluorescence (× 380).

We have looked for other possible explanations of these findings but none have been found. A genetic basis for the presence of this antibody to osteosarcoma is excluded by the finding of an increased incidence and titer of antibody in serums from the friends of one osteosarcoma patient. The mean ages of the family members (32.5 years) was not sufficiently different from the mean ages of the control series of normal blood-bank donors (36.4 years) to explain the increased incidence and titers of the antibodies. Also, differences in sex and blood type between the four groups were not adequate to explain these findings. The possibility that these observations are the result of the interaction of isoantibodies and their respective antigens is excluded by the fact that each osteosarcoma patient's serum reacted strongly with his own osteosarcoma as well as with the osteosarcomas from other patients. Furthermore, the blocking experiments, in which reactive unlabeled serums were found to block the fixation of fluorescein-labeled globulins from patients B and C with autologous osteosarcoma cells, exclude isoantigens and isoantibodies as a possible explanation of these findings.

The 29 percent reactivity in normal serums against these osteosarcomas would appear to indicate that this infectious agent frequently produces unrecognized infections of normal individuals. Similar epidemiological studies of normal animals have frequently revealed antibodies to naturally occurring oncogenic viruses in the chicken (*2*) (Rous sarcoma and leukosis virus) and mouse [polyoma (*3*) and Gross leukemia virus (*4*)]. Likewise, it might be expected that some normal human serums, in the absence of recognizable neoplastic disease, might contain antibodies against antigens associated with human neoplasms.

The significance of these findings in relation to the etiology of osteosarcomas remains to be determined. The etiology of this disease is unknown, but a genetic origin has been suggested by reports of the remarkable familial occurrence of this neoplasm (*5*). As many as four siblings in the same family have been afflicted (*6*). How-

ever, Roberts and Roberts (*7*) suggested the possible adjuvant role of an infectious agent in the etiology of osteosarcomas in reporting the simultaneous development of this neoplasm in three members (one brother and two sisters) of the same family. Additional clinical evidence consistent with a viral etiology of osteosarcoma are reports of the simultaneous occurrence of osteosarcomas at multiple sites in the same patient (*8*) and the predilection of this disease for young people.

All animal neoplasms induced by the same virus have been found to contain a common tumor antigen (*9*). It is possible that the presence of a common antigen in human osteosarcomas demonstrated by this study similarly indicates a common virus etiology. A viral etiology is also suggested by the recent isolation of a murine osteosarcoma virus that induces osteosarcomas in mice which have many similarities to the human disease (*10*).

This study suggests the close association of an infectious agent with human osteosarcomas. Whether this agent is an incidental passenger in these neoplasms or related to their etiology remains to be determined. However, the clinical evidence in favor of a viral etiology, when combined with the implications of this study, strongly suggests that attempts should be made to isolate a viral agent from this neoplasm.

DONALD L. MORTON

Surgery Branch,
National Cancer Institute,
National Institutes of Health,
Bethesda, Maryland 20014

RICHARD A. MALMGREN

Pathologic Anatomy Branch,
National Cancer Institute

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Human Monocytes: Distinct Receptor Sites for the Third Component of Complement and for Immunoglobulin G

Abstract. *Human monocytes contain two distinct receptor sites, one specific for the third component of complement (C'3), the other for immunoglobulin G (γG). The two receptors may function either independently or cooperatively in the induction of phagocytosis. Ingestion of erythrocytes coated with immunoglobulin M antibody requires a relatively large number of bound C'3 molecules per cell. Ingestion of erythrocytes sensitized with γG antibody is independent of complement; however, the reaction is inhibited by concentrations of γG far below those in normal serum. Inhibition by γG-globulin is overcome by a relatively small number of bound C'3 molecules per cell. The two monocyte receptors exert a cooperative effect on ingestion by monocytes of erythrocytes coated with γG antibody in the presence of inhibitory amounts of free γG.*

Human monocytes contain a receptor site for immunoglobulin G (γG) (*1*, *2*) which facilitates attachment and then ingestion of red cells coated with γG antibodies. This receptor is present not only on monocytes from the peripheral blood, but also on macrophages (*2*, *3*). The reaction between monocytes or macrophages and red cells sensitized with γG antibody occurs in vitro in the absence of complement (*1–3*). These γG receptors, however, bind specifically

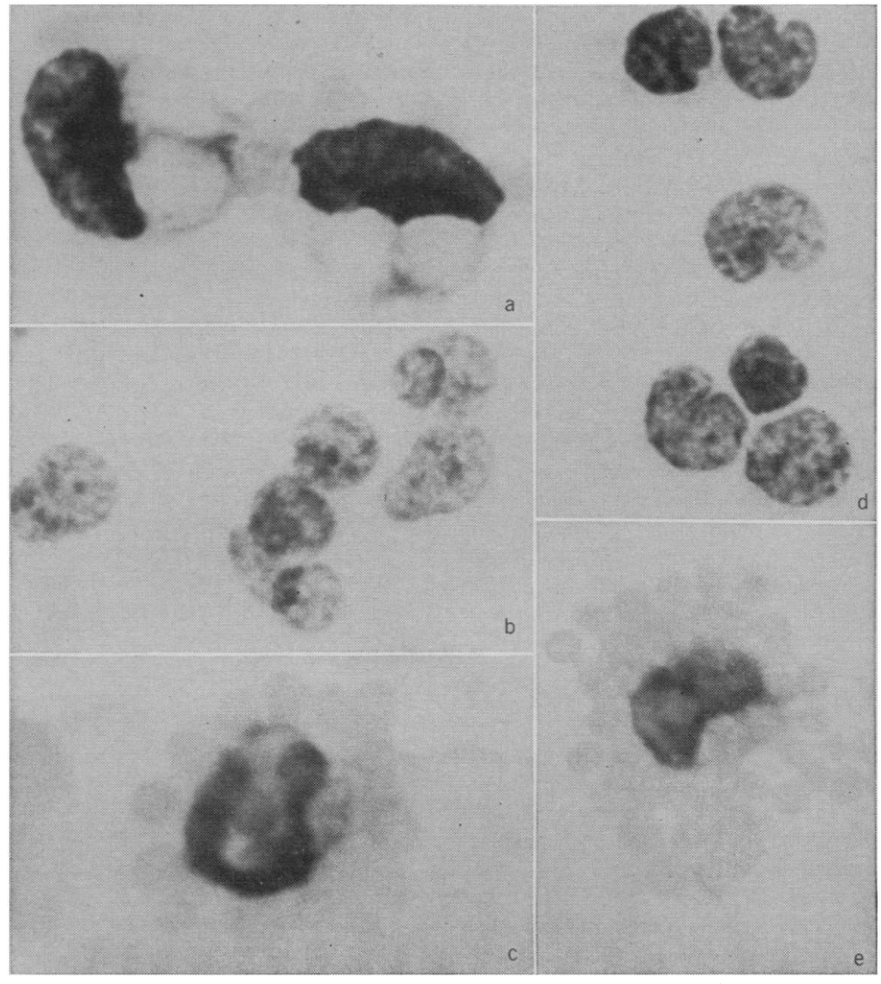

Fig. 1. Effect of antibody type and complement on interaction of isolated monocytes with sensitized sheep erythrocytes. (a) Positive reaction with erythrocytes sensitized with γG antibody in the absence of complement; (b) inhibition of reaction (a) by γG-globulin in medium (1 mg/ml); (c) neutralization of inhibition (b) by erythrocyte-bound complement (C'1,4,2,3); (d) negative reaction with erythrocytes sensitized with γM antibody in the absence of complement; (e) positive reaction with erythrocytes (d) after their reaction with complement components C'1, C'2, and C'3.