Mixed Leukocyte Reactivity and Leukemia: Study of Identical Siblings

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ABSTRACT This report describes a search for tumor-specific transplantation antigens in man by testing four sets of identical siblings using the mixed leukocyte transformation reaction. In each case, one member of the set had acute leukemia in relapse. In no instance did the leukemic cells of the patient stimulate the cells of the normal twin, nor did cells from the normal twin stimulate cells from the leukemic patient. Possible explanations for the failure to detect a leukemia-associated antigen in these studies are discussed.

INTRODUCTION

The availability of inbred animals has made it possible to study tumor-specific antigens in mice. Immunization of a normal mouse with tumor from an isogeneic mouse provided the basis for demonstration of a unique tumor-specific transplantation antigen (TSTA) for each chemically induced tumor and a common TSTA for tumors induced by a given virus (1). Recent reports suggest that human tumors, like animal tumors, may have TSTA (2-5). Sets of identical twins, one of whom has leukemia, provide an opportunity for investigating TSTA in an isogeneic system in man. Immunization of the normal twin with irradiated or formalin-treated leukemic cells from the identical sibling would be most informative but would carry an unacceptable risk of transmission of leukemia. An *in vitro* test that may be useful in detecting TSTA is the mixed leukocyte transformation reaction (6). Reactivity in mixed leukocyte culture depends on strong antigenic differences such as those controlled by the major histocompatibility locus in man (7). This report describes a search for TSTA in man by testing four pairs of identical twins by mixed leukocyte culture. In each case, one member of the pair had acute leukemia in relapse.

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METHODS

Approximately 30 ml of whole blood were drawn under sterile conditions into a syringe containing 0.3 ml of heparin.¹ The blood was centrifuged in sterile polystyrene tubes² at 250 *g* for 15 min. Some cell-free supernatant plasma was removed and retained for later use as medium supplement. The remaining plasma and buffy coat and a small portion of the adjacent erythrocyte layer were transferred to a sterile 17 × 100 mm disposable polystyrene tube and were allowed to sediment at 37°C for 60-120 min. The supernatant leukocyte-rich plasma was removed and diluted with Eagle's minimum essential medium modified for suspension culture.³ To each 100 ml of medium we added 1.0 ml of nonessential amino acids, 0.6 ml of 5% L-glutamine, 10,000 U of penicillin, and 10 mg of streptomycin. The medium was supplemented with 15% autologous cell-free plasma. In those cases in which the patient had recently been treated with chemotherapy, the leukocytes were washed three times in the tissue culture medium before suspension in a medium containing 15% cell-free plasma from an unrelated individual. Lymphocytes to be used as responding cells were adjusted to a concentration of 5 × 10⁶ lymphocytes per milliliter. Leukocytes to be used as stimulating cells were adjusted to concentrations varying from 1 to 4 × 10⁶ leukocytes per ml and irradiated with 2500 R from a ⁶⁰Co source at a dose rate of 125 R/min. Irradiation made the cells incapable of synthesizing significant amounts of deoxyribonucleic acid, yet they could stimulate other cells to do so (8). Duplicate cultures were prepared in polystyrene tubes, each containing 2 ml of responding cells and 2 ml of stimulating cells. The cell suspensions were incubated for 6 days at 37°C under an atmosphere of humidified 5%CO₂ in air. At the end of 6 days, 4 μCi of tritiated thymidine (SA 6.7 Ci/mM) was added to each culture. 3 hr later the cells were centrifuged and, after the removal of the supernatant, 5 ml of cold 5% trichloroacetic acid was added to each culture. The resulting precipitate was washed three times with cold 5% trichloroacetic acid, solubilized overnight in hydroxide of Hyamine,⁴ and counted in a liquid

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² Falcon Plastics, Div. of Bioquest, Los Angeles, Calif.

³ Grand Island Biological Co., Grand Island, New York.

⁴ Packard Instrument Co., Downers Grove, Ill.

TABLE I
Tritiated Thymidine Uptake per Culture in Mixtures of Human Isogenic Leukocytes

Sibling set	Type leukemia	% Blasts in patient's stimulating mixture	Cell mixture*	Concentration of stimulating cells ($\times 10^6/\text{ml}$)	<i>cpm</i>	<i>P</i> †
I§	Acute lymphoblastic leukemia	20	AA _X	1.0	1401	
			AB _X	1.0	2109	NS
			AC _X	1.0	2424	NS
			AU _X	1.0	16,799	<0.01
			BB _X	1.0	2851	
			BA _X	1.0	764	NS
			BU _X	1.0	38,762	<0.01
			CC _X	1.0	222	
			CA _X	1.0	354	NS
			CU _X	1.0	24,086	<0.01
			UU _X	1.0	625	
			UA _X	1.0	35,915	<0.01
			UB _X	1.0	59,160	<0.01
			UC _X	1.0	58,916	<0.01
II	Acute lymphoblastic leukemia	24	AA _X	1.0	1230	
			AB _X	1.0	802	NS
			AB _X	2.0	863	NS
			AU _X	1.0	10,313	<0.01
			BB _X	1.0	857	
			BA _X	1.0	1009	NS
			BA _X	2.0	1151	NS
			BU _X	1.0	41,829	<0.01
			UU _X	1.0	872	
			UA _X	1.0	31,361	<0.01
			UB _X	1.0	13,429	<0.01
III	Acute myeloblastic leukemia	25	AA _X	1.0	429	
			AB _X	1.0	272	NS
			AB _X	1.5	212	NS
			AB _X	2.0	248	NS
			AU _X	1.0	14,525	<0.01
			BB _X	1.0	295	
			BA _X	1.0	309	NS
			BA _X	2.0	212	NS
			BU _X	1.0	27,568	<0.01
			UU _X	1.0	369	
			UA _X	1.0	25,401	<0.01
			UB _X	1.0	18,078	<0.01
IV	Acute myeloblastic leukemia	80	AA _X	1.0	666	
			AB _X	1.0	457	NS
			AU _X	1.0	5378	<0.05
			BB _X	1.0	1014	
			BA _X	1.0	859	NS
			BA _X	4.0	507	NS
			BU _X	1.0	21,589	<0.01
			UU _X	1.0	1078	
			UA _X	1.0	37,536	<0.01
			UB _X	1.0	46,263	<0.01

* A, patient; B and C, identical siblings; U, unrelated individual.

† *P*, probability that mixture is different from the isogenic control by chance alone; NS, not significant.

§ Sibling group I, identical triplets.

|| Subscript X indicates irradiated cells.

scintillation spectrometer. All values were corrected for quenching by automatic external standardization and for background.

Leukemic blast cells from the peripheral blood of two patients were stored by freezing. Leukocyte and blast cell-rich plasma was obtained as above and was added to a mixture of Eagle's medium and dimethyl sulfoxide (DMSO). The final concentration of the mixture was 70% Eagle's medium, 20% autologous plasma, and 10% DMSO. This mixture was distributed into sterile polystyrene tubes and frozen to -25°C at a rate of $1^{\circ}\text{C}/\text{min}$. The cells were stored at -80°C . When subsequently used, the frozen cells were thawed rapidly in a 37°C water bath and washed three times in Eagle's medium containing 15% cell-free plasma from an unrelated individual. The cells were then adjusted to concentrations of from $1-2 \times 10^6/\text{ml}$, irradiated with 2500 R as above, and used as stimulating cells in mixed leukocyte culture.

The lymphocytes of all twin pairs were serotyped with cytotoxic antisera according to the method of Amos (9). The lymphocytotoxic antisera used in these experiments were obtained from the N.I.H. Serum Bank (Bethesda, Md.) and from Dr. Bernard Amos (Duke University, Durham, N. C.).

One-way analysis of variance was performed within each experiment to evaluate means of duplicates relative to duplicate variability. All pairs of means were tested by the Tukey procedure (10).

RESULTS

Each sibling set was similar in appearance, and dermatoglyphic study indicated identity. In three sets, identity was confirmed by determination of 14 blood genetic markers. In set IV, blood genetic markers were not studied because of recent multiple transfusions. Each sibling set showed an identical phenotype with well-defined antisera recognizing the major HL-A antigens. In set III family analysis showed identical HL-A genotypes of the twins.

The results of testing the four sets of siblings using a mixed leukocyte culture are presented in Table I. The activity in counts per minute represents means of duplicate cultures. Reproducibility was such that the average deviation of a duplicate from its mean was 13.5%. Controls with cells from unrelated individuals are shown in Table I. Controls involving mixtures of irradiated cells in all combinations did not show a significant uptake of thymidine. In no instance did the leukemic cells of the patient stimulate the cells of the normal twin, nor did cells from the normal twin stimulate cells from the leukemic patient. Careful washing of the lymphocytes in an attempt to eliminate any serum factors which might abrogate an in vitro immune reaction did not alter these results.

Sibling set I consisted of identical triplets and was studied on three additional occasions. The percentages of blast cells in the patient's stimulating mixture in these subsequent experiments were 46, 8, and 33. The results were similar to those presented in Table I. Twin pair III

TABLE II
Tritiated Thymidine Uptake in Mixed Leukocyte Culture

Sibling set	% Blasts in patient's stimulating mixture	Cell mixture*	Concentration of stimulating cells ($\times 10^6/\text{ml}$)	<i>cpm</i>	P†
I	38	AAx§	1.0	4171	
		AFx	1.0	1834	NS
		AFx	2.0	4114	NS
		AUx	1.0	22,318	<0.01
		BBx	1.0	434	
		BFx	1.0	580	NS
		BFx	2.0	346	NS
		BUx	1.0	22,645	<0.01
		CCx	1.0	642	
		CFx	1.0	747	NS
		CFx	2.0	706	NS
		CUx	1.0	21,694	<0.01
		UUx	1.0	2504	
		UFx	1.0	10,891	<0.01
		UFx	2.0	20,517	<0.01
III	53	AAx	1.0	197	
		AFx	1.0	219	NS
		AFx	2.0	239	NS
		AUx	1.0	1347	<0.01
		BBx	1.0	210	
		BFx	1.0	249	NS
		BFx	2.0	233	NS
		BUx	1.0	7488	<0.01
		UUx	1.0	653	
		UFx	1.0	47,412	<0.01
		UFx	2.0	48,047	<0.01

* A, human irradiation chimera; B and C, identical siblings; U, unrelated individual; F, patient's peripheral leukocytes frozen and stored prior to total body irradiation.

† P, probability that mixture is different from the isogeneic control by chance alone; NS, not significant.

§ Subscript X indicates irradiated cells.

also was studied on two other occasions. No interaction occurred between the cells of the leukemic and normal twin, even though stimulating cell concentrations as high as $2.8 \times 10^6/\text{ml}$ with 35% blasts were used.

On two occasions peripheral blood leukocytes, including leukemic blast cells, were stored by freezing them before treatment of the patient with 950–1000 rads total body irradiation and isogeneic marrow grafting. $3\frac{1}{2}$ wk and 14 wk, respectively, after engraftment these frozen cells were thawed, irradiated, and used as stimulating cell mixtures. The results of these experiments are presented in Table II. The frozen cells did not stimulate the peripheral blood lymphocytes from the chimera or the normal twin, but they did stimulate allogeneic cells.

Two additional experiments were performed (twin pairs II, III) to evaluate the possibility of destruction of leukemia-associated antigen by irradiation. In these experiments, cells from the twin pair were cultured together without irradiation of either cellular component.

In these bidirectional interactions there was no instance of tritiated thymidine uptake greater in the mixture than the sum of isotope uptake in each individual's cells cultured alone.

DISCUSSION

Cells from normal identical twins do not interact in mixed leukocyte culture (11). Similarly, the present study showed no interaction in mixed leukocyte culture in four sets of identical siblings, one of whom had leukemia. In several experiments, the cells of the patient were carefully washed, and autologous plasma was replaced with plasma from an unrelated individual in order to remove any residual inhibitor due to chemotherapy and to attempt to eliminate any serum factors which might abrogate an in vitro immune reaction. These negative results are not in agreement with two recent reports. Bach, Bach, and Joo, using the mixed leukocyte test, detected two instances of nonreciprocal stimulation between sibling pairs (12). In each case, the individual whose cells were stimulatory had acute leukemia. Normal human siblings, matched at the HL-A locus, were always reciprocally nonstimulatory. Thus, the results suggested that the cells of the leukemic patient might possess leukemia-associated antigens. Unfortunately, cytotoxicity typing of these patients and their families was not reported, and therefore HL-A identity between the involved siblings could not be established. Fridman and Kourilsky studied nine cases of acute leukemia (13). Leukemic blast cells were frozen during relapse and later, during remission, mixed leukocyte cultures were studied between the frozen cells and the patient's normal lymphocytes. Stimulation was observed in six of nine instances by radioautography and in four of seven instances by uptake of tritiated thymidine. The results suggested that in vivo presensitization may be necessary to demonstrate a leukemia-associated antigen in the mixed leukocyte culture test. In contrast to these results, leukemic blast cells from two of our patients were stored by freezing before treatment of the patient with total body irradiation and isogeneic marrow transplantation. After engraftment, the transplanted lymphocytes were exposed in vivo to previously stored host tumor cells which had been given 10,000 R and then had been injected subcutaneously into the patient at weekly intervals. After 3½ and 14 wk, respectively, mixed leukocyte reactivity was tested using the original frozen host leukemic cells. Despite the presumed immune status of the grafted lymphocytes, the irradiated leukemic blast cells did not stimulate the peripheral blood lymphocytes of the chimeric patients or of the normal twins, but did stimulate allogeneic cells.

There are, at least, the following three possible explanations for our failure to detect a leukemia-associated

antigen in these studies: (a) there may be no antigen uniquely associated with human leukemia; (b) human leukemia-associated antigens may exist but may not be of sufficient immunogenicity to elicit a response in mixed leukocyte culture; (c) if acute leukemia is a virus-induced disease in man, a normal twin may have an altered immune state through exposure to the common agent. Thus, he might be tolerant to the agent in a fashion similar to the tolerance observed in mouse breast carcinoma (14, 15). At present, 6-12 months after study, there is no evidence of leukemia in any of the normal identical siblings.

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REFERENCES

1. Houghton, G., and D. B. Amos. 1968. Immunology of carcinogenesis. *Cancer Res.* **28**: 1839.
2. Hellström, I., K. E. Hellström, G. E. Pierce, and A. H. Bill. 1968. Demonstration of cell-bound and humoral immunity against neuroblastoma cells. *Proc. Nat. Acad. Sci. U. S. A.* **60**: 1231.
3. Morton, D. L., and R. A. Malmgren. 1968. Human osteosarcomas: immunologic evidence suggesting an associated infectious agent. *Science (Washington)*. **162**: 1279.
4. Klein, G., E. Klein, and P. Clifford. 1967. Search for host defences in Burkitt lymphoma: membrane immunofluorescence tests on biopsies and tissue culture lines. *Cancer Res.* **27**: 2510.
5. Gold, P., and F. O. Freedman. 1965. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J. Exp. Med.* **121**: 439.
6. Report of a WHO Scientific Group. 1966. Immunotherapy of cancer. *World Health Organ. Tech. Rep. Ser. No.* **344**.
7. Bach, F. H., and D. B. Amos. 1967. Hu-1: major histocompatibility locus in man. *Science (Washington)*. **156**: 1506.
8. Kasakura, S., and L. Lowenstein. 1967. Irradiated and preserved leukocytes in mixed leukocyte cultures. *Proc. Soc. Exp. Biol. Med.* **125**: 355.

9. Amos, D. B., H. Bashir, W. Boyle, M. MacQueen, and A. Tiilikainen. 1969. A simple microcytotoxicity test. *Transplantation*. 7: 220.
10. Snedecor, G. W. 1956. Statistical Methods. Applied to Experiments in Agriculture and Biology. The Iowa State College Press, Ames, Iowa. 5th edition. 237-256.
11. Bain, B., M. R. Vas, and L. Lowenstein. 1964. The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood*. 23: 108.
12. Bach, M. L., F. H. Bach, and P. Joo. 1969. Leukemia-associated antigens in the mixed leukocyte culture test. *Science (Washington)*. 166: 1520.
13. Fridman, W. H., and F. M. Kourilsky. 1969. Stimulation of lymphocytes by autologous leukaemic cells in acute leukaemia. *Nature (London)*. 224: 277.
14. Morton, D. L. 1966. Acquired immunological tolerance and carcinogenesis by the mammary tumor virus. *Surg. Forum*. 17: 107.
15. Lavrin, D. H., P. B. Blair, and D. W. Weiss. 1966. Immunology of spontaneous mammary carcinomas in mice. IV. Association of mammary tumor virus with the immunogenicity of C3H nodules and tumors. *Cancer Res*. 26: 929.