standard Ringer solution were used in the third experiment. They were hypertonic Ringer solutions (343 milliosmole/liter) which were made by the addition of 17 m*M* of mannose, glucose, sucrose, or NaCl to standard Ringer solution. These test solutions also yielded an increase in the discharge rate (Fig. 3). When the perfusion pressure was suddenly increased by 20 cm-H_2O (Fig. 3), no change in the discharge rate was observed. I conclude that increase in discharge rate is in accord with increase in osmotic pressure in the perfusion solutions.

Sometimes a decrease in osmotic pressure, as well as an increase, caused an increase in discharge rate in multifiber preparations. Jacobs and Adachi (*3*) report osmoreceptors in the liver of the rat which are activated by hypotonic solutions. The same type of receptors thus occur in the guinea pig liver as well.

I suggest that osmoreceptors in the liver monitor osmotic pressure of the portal venous blood. They are linked to the central nervous system through the hepatic branch of the vagal nerve. These receptors may represent the afferent limb of reflex systems involved in the control of body fluid.

A. NIIJIMA

Department of Physiology,
Niigata University
School of Medicine, Niigata, Japan

References and Notes

1. F. R. S. Verney, *Proc. Roy. Soc. Ser. B Biol. Sci.* **135**, 25 (1947).
2. F. J. Haberich, O. Aziz, P. E. Nowacki, *Arch. Ges. Physiol.* **285**, 73 (1965); F. J. Haberich, *Fed. Proc.* **27** (5), 1137 (1968).
3. H. Jacobs and A. Adachi, personal communication.

4 June 1969; revised 62 August 1969

Leukemia-Associated Antigens in the Mixed Leukocyte Culture Test

Abstract. *Patients with acute leukemia (blast cells in the peripheral blood) manifest antigens in the one-way mixed leukocyte culture test. Leukocytes from normal patients and leukocytes of patients in remission do not clearly show these antigens. These antigens could be leukemia-associated antigens in man.*

Tumor-associated transplantation antigens—antigens associated with tumor cells but not on the animal's normal tissue—have been demonstrated in several animal species (*1*). These antigens were so named because they were detected by transplantation techniques. In order to detect antigens associated with tumor cells in man, one is restricted to immunological methods other than direct transplantation techniques. Consequently, evidence of tumor-associated antigens is more limited in man.

Using the indirect fluorescent antibody assay, Klein *et al.* (*2*) showed that on Burkitt lymphoma cells there was an "antigen" associated with the lymphoma cells and that this antigen was not detectable on normal bone marrow cells of the tested patient. Malmgren and Morton (*3*) observed antibodies in the serums of melanoma patients which gave relatively specific fluorescence with melanoma cells. Gold *et al.* (*4*), using immunoelectrophoresis, reported that carcinoma cells from the gastrointestinal tract possess antigenic determinants that are absent from normal cells of adult origin but are present in embryonic tissues and tumors from the gastrointestinal tract. Hellstrom *et al.* (*5*), using the colony inhibition assay, demonstrated tumor-associated antigens on neuroblastomas and some gastrointestinal tumors.

We now report results of mixed leukocyte culture tests with human leukemic leukocytes in vitro. Our data indicate that leukemic cells carry, and manifest in mixed leukocyte culture tests, antigens which normal lymphocytes do not manifest. These antigens could be leukemia-associated antigens.

In the mixed leukocyte culture test, peripheral blood leukocytes of one individual (A) are mixed with leukocytes (treated with mitomycin-C and designated subscript "m") of another individual (B). The untreated responding cells (A) enlarge and divide in response to foreign histocompatibility antigens present on the treated stimulating cells (B_m). After the cells are cultured for 7 days, incorporation of tritiated thymidine into acid-precipitable material is studied in allogeneic (AB_m) and isogeneic (AA_m) cell mixtures. The enhanced incorporation of tritiated thymidine in allogeneic mixtures (stimulation in mixed leukocyte culture test) is a measure of the response of A cells to foreign histocompatibility antigens of B_m cells and reflects the antigenic disparity of the two individuals at HL-A—the major histocompatibility locus in man (*6*). If the two test cells are identical at HL-A, then the cell mixtures AB_m and BA_m will not incorporate thymidine at a rate significantly higher than their respective controls, AA_m and BB_m (nonstimulation, identity in the mixed leukocyte culture test). If a mixture AB_m shows no stimulation, this result will only be accepted as indicating identity if, in suitable controls in the same experiment, the test cells (A) react as responding cells and the treated cells (B_m) act as stimulating cells.

In the human population, HL-A is a highly polymorphic locus, and thus individuals are generally heterozygous at this locus; two unrelated individuals will usually be heterozygous for different alleles. In a typical family, we can thus arbitrarily assign alleles *a* and *b* to the father and *c* and *d* to the mother. Segregation of these alleles yields four different combinations (*ac*, *ad*, *bc*, and *bd*) in the siblings, and 25 percent of ibling pairs should be identical at HL-A. Two such identical siblings will be heterozygous for the same alleles and will thus be identical at HL-A. Any siblings with dissimilar alleles will be reciprocally HL-A disparate. No sibling pairs should thus stimulate in one direction but not in the other when both parents are unlike heterozygotes. Consonant with these considerations, 28.2 percent of sibling pairs studied failed to stimulate in mixed leukocyte culture tests (*6*), and all of the nonstimulatory cell mixtures were nonstimulatory in both directions. No nonreciprocal stimulating pairs were found among siblings tested.

Using the mixed leukocyte culture test to choose donors for bone marrow transplantation we have studied families of 36 leukemic patients. Of these families, 30 were studied when the patient was in remission, that is, no leukemic cells appeared in a smear of the peripheral blood. In these families we found sibling pairs identical in the mixed leukocyte culture test in a frequency not significantly different from that found in normal sibling pairs (*6*). In all of the sibling pairs in these families that were identical in the mixed leukocyte culture test, there was no exception to the rule that the siblings were nonstimulatory in both directions. Six families with a leukemic member were studied at a time when the patient was in relapse, that is, between 30 and 100 percent of the leukocytes in a smear of peripheral blood

were leukemic blast cells. In three of these families there was reciprocal stimulation in all patient-sibling pairs tested; this is consistent with findings in the normal population indicating that the patients had no siblings identical in the mixed leukocyte culture test.

In each of the remaining three families, cells of the patient did not respond to the cells of one sibling. In one case, the patient had a sibling, identical in the mixed leukocyte culture test, who showed reciprocal nonstimulation as in normals. In the other two cases, the cells of the patient showed nonreciprocal stimulation with the cells of one sibling. In both of these unusual cases cells of the leukemic patient were stimulatory to cells of one of his siblings, but cells of the sibling were not able to stimulate his cells. Appropriate controls with cells from unrelated individuals demonstrated that the leukemic test cells could both stimulate and respond to allogeneic cells (Table 1).

In experiment 1, Table 1, cells of the patient A.T. cultured alone incorporated 593 count/min of tritiated thymidine. In mixture with stimulatory cells treated with mitomycin-C of the sibling at three concentrations of stimulating cells, between 572 and 796 count/min were incorporated, that is, no significant stimulation. Several concentrations of stimulating cells were used (*7*). Mixture AC_m verified that the patient's cells can respond to the stimulating cells of another sibling (14,672 count/min as compared with a control of 593 count/min); mixture XB_m verified that the cells treated with mitomycin-C (B_m) of the sister can stimulate allogeneic cells (3816 count/min incorporated compared with the control of X cells cultured alone—472 count/min). When the cells of the sister (B) were tested for their response to the stimulating cells (A_m) of the patient, there was clear stimulation.

In experiment 2, cells of this patient were tested with the cells of the same sibling. The ability of the cells of A.T. to stimulate his sister's cells is again shown here (9933 to 37,825 count/min incorporated in experimental mixtures as opposed to a control of 2035 count/min). The cells of the patient were tested when no normal lymphocytes were found on smear. We thus did not attempt to test the response of the patient's lymphocytes to allogeneic stimulating cells.

In experiment 3, cells of a second patient, also called A, and cells of his normal sibling B both incorporate about 1200 count/min in the control cultures, as do cells of an unrelated individual X. In the mixture AB_m, at two different concentrations of stimulating cells, there is no significant stimulation. In the other direction, however, the cells of the normal sibling B did respond to the stimulating cells of the leukemic patient. The necessary control mixtures are AX_m and XB_m; they demonstrate that the cells of the leukemic patient are able to respond to an allogeneic stimulus and that the cells of the normal sibling are able to stimulate allogeneic cells. Experiment 4 confirms the results of experiment 3.

This nonreciprocal stimulation between siblings in two out of three families with a leukemic member, wherein cells of the patient failed to respond to cells of his siblings, is in sharp contrast to results obtained in families with no leukemic members. In the latter group, of 291 sibling pairs tested, cells of 75 pairs were reciprocally nonstimulatory, and no sibling pairs showed nonreciprocal stimulation. The probability of finding these two cases in the three families with a leukemic member tested, by chance alone, with no cases in the normal families, is 0.008—establishing the significance of these findings in relation to these patients. The probability, calculated by the Fisher exact test, is a conservative estimate both on the basis of the statistical test used and as only the 75 pairs of reciprocal nonstimulators are considered.

Nonreciprocal stimulation might be the trivial expression of the presence of contaminating leukocytes in the patients' blood from previous blood transfusions. This is unlikely on two grounds. (i) We have tested more than 40 potential kidney transplant recipients, all of whom had had multiple transfusions, and we have never observed this phenomenon; and (ii) experiment 2 (Table 1) demonstrates the stimulatory ability of the blast cells of the patient at a time when the patient had no normal lymphocytes or polymorphonuclear leukocytes on his smear.

There are at least three possible interpretations of these findings that invoke special antigenic properties of leukemic cells. (i) The cells of the leukemic patients may possess leukemia-associated antigens which are stimulatory in the mixed leukocyte cuture test. (ii) Leukemic cells may express their minor loci antigens (non HL-A) more strongly, and, in these cases, disparity at minor loci can result in stimulation in mixed culture. (iii) Blast cells may show their own unique antigens (*8*).

It is of interest that the antigens associated with acute leukemia can elicit a response in the mixed leukocyte culture reaction since only strong histocompatibility differences appear to initiate a response (*6*). They consequently behave as if they were "strong" antigens. The strength of the antigens, whether they are modifications of the

Table 1. Experiments 1 and 2 are studies of family of patient A.T., a 34-year-old male with acute lymphoblastic leukemia. A, patient; B, sister; C, another sibling; X, unrelated individual. Experiments 3 and 4 are studies of family of patient C.A., a 15-year-old male with acute granulocytic leukemia. A, patient; B, brother; X, unrelated individual. Responding cell concentrations are 0.3×10^6 mononuclear cell/ml for healthy siblings and unrelateds, 0.75×10^6 mononuclear cell/ml for patients' cells. Stimulating cell concentrations presented refer to total leukocytes per milliliter. Mixed cultures are performed as described (*11*). Leukocytes from the test individuals are incubated for 7 days at 37°C and, on day 7, 2 μc of tritiated thymidine is added to each culture for 5 to 5.5 hours. Count/min refers to the average for washed acid precipitates of replicate cultures.

Cell mixture	Concentration of stimulating cells (No. $\times 10^{-6}$/ml)	Activity (count/min)	P*
	Experiment 1		
A		593	
AB_m	0.5	572	$>.5$
AB_m	1.0	696	$>.5$
AB_m	1.5	796	$>.5$
B		442	
BA_m	0.5	2832	$<.05$
BA_m	1.0	53793	$<.02$
AC_m	0.5	14672	$<.02$
X		472	
XB_m	0.5	3816	$<.05$
	Experiment 2		
B		2035	
BA_m	0.5	9933	$<.05$
BA_m	1.0	20061	$<.02$
BA_m	1.5	37825	$<.02$
	Experiment 3		
A		1281	
AB_m	0.5	874	$>.5$
AB_m	1.0	1338	$>.5$
B		1195	
BA_m	0.5	5403	$<.05$
BA_m	1.0	9209	$<.02$
AX_m	1.0	33053	$<.02$
X		1291	
XB_m	0.5	22310	$<.02$
	Experiment 4		
A		3037	
AB_m	0.5	2915	$>.5$
AB_m	1.0	5201	$>.4$
AB_m	1.5	5019	$>.4$
B		1070	
BA_m	0.5	1664	$>.4$
BA_m	1.0	7283	$<.02$
AX_m	0.5	33254	$<.02$
X		841	
XB_m	0.5	42205	$<.02$

* P = probability that a given allogeneic mixture is different from the isogeneic control by chance alone.

antigens of the HL-A system or simply other strong antigens, is of importance in considering possible cellular immunotherapy. It has been demonstrated that bone marrow transplantation between animals who are well matched at the major histocompatibility locus will not result in early, fatal, graft-versus-host reactions (*9*). Evidence for this has also been obtained in man (*10*). Thus one might hope to transplant into a leukemic patient immunologically competent normal cells which, while well matched for the normal tissue of a patient, would respond to the "strong" antigens of the leukemic tissue and specifically destroy these cells.

MARILYN L. BACH
Department of Medical Genetics, University of Wisconsin, Madison 53706

FRITZ H. BACH
Departments of Medical Genetics and Medicine, University of Wisconsin

PATRICIA JOO
Department of Pediatrics, University of Wisconsin

References and Notes

1. K. E. Hellstrom and I. Hellstrom, *Advan. Cancer Res.*, in press; G. Klein, *Fed. Proc.*, in press.
2. G. Klein, E. Klein, P. Clifford, *Advan. Cancer Res.* **27**, 2510 (1967).
3. R. A. Malmgren and D. L. Morton, *Science* **162**, 1279 (1968).
4. P. Gold, M. Gold, S. O. Freedman, *Advan. Cancer Res.* **28**, 1331 (1968).
5. I. Hellstrom, K. E. Hellstrom, G. E. Pierce, A. H. Bill, *Proc. Nat. Acad. Sci. U.S.* **60**, 1231 (1968).
6. F. H. Bach, *Progress in Medical Genetics*, A. G. Steinberg and A. G. Bearn, Eds. (Grune and Stratton, New York, 1969), vol. 6, p. 201.
7. R. J. Albertini and F. H. Bach, *J. Exp. Med.* **128**, 639 (1968).
8. We thank G. Klein and O. Smithies for discussion of these results.
9. D. E. Uphoff, *J. Nat. Cancer Inst.* **19**, 123 (1957); M. Simonsen, *Progr. Allergy* **6**, 349 (1962); R. A. Good, C. Martinez, A. E. Gabrielsen, *Advan. Pediat.* **13**, 93 (1964); E. D. Thomas, R. Storb, R. B. Epstein, R. H. Rudolph, *Transplant. Proc.* **1**, 31 (1969).
10. F. H. Bach, R. J. Albertini, J. L. Anderson, P. Joo, M. M. Bortin, *Lancet* **1968-II**, 1364 (1968); R. A. Gatti, H. J. Meuwissen, H. D. Allen, R. Hong, R. A. Good, *ibid.*, p. 1366.
11. F. H. Bach and N. Voynow, *Science* **153**, 545 (1966).
12. Supported by NIH grants GM 15422 and AI 08439 and ONR contract N00014-67-0128-0003. Paper No. 1318 from the Laboratory of Genetics, University of Wisconsin, Madison. We thank M. Hutchinson and C. Kurtyka for technical assistance. F.H.B. is an established investigator of the American Heart Association.

31 July 1969

Localization of Antigenic Determinants in the Polypeptide Chains of Collagen

Abstract. *After being injected with collagen from rat or guinea pig skin, rabbits form high titer, species-specific antibodies to collagen. Antibody is directed primarily against the α2 chain of collagen with little reaction with the α1 chain. The amino terminal peptide of cyanogen bromide cleavage of the α2 chain of guinea pig skin collagen, α2-CBl, effectively inhibited the antigen-antibody reaction, an indication that a major antigenic determinant of collagen is located at the amino terminal of the α2 chain.*

Although collagen is immunogenic (*1*) the location of the antigenic determinants in the molecule and the degree of the helical structure required for recognition by antibody molecules are unknown. For example, Schmitt *et al.* (*2*) and Davison *et al.* (*3*) reported that the complement-fixing activity of collagen with rabbit antiserums decreased when the collagen was denatured. However, treatment of collagen with proteases also decreased the antibody binding activity. The major effect of protease treatment is the removal of the amino terminal portions of the collagen molecule, which differs in composition from the rest of the molecule and is probably not helical (*4*). Steffen *et al.* (*5*), using gelatin-coated red blood cells, determined the various specificities of antibodies to collagen. They could distinguish antibodies that were not species-specific, antibodies that were species-specific, and antibodies that were directed to pepsin-labile structures of collagen. LeRoy (*6*) separated the components of denatured collagen by chromatography on carboxymethyl cellulose and found that the α and β components had no antibody binding activity whereas the material not binding to the column was active. Thus from LeRoy's study the antigenic determinant appeared to be separable from the chains that form the collagen molecule.

In part, these differences may be due to technical difficulties encountered in assaying for antibody with both native and denatured collagen. Since we have found that both native and denatured collagen precipitate with antibody to collagen (*7*), we are now reporting on the distribution of antigenic determinants in the α and β components isolated from denatured collagen.

Salt-soluble lathyritic collagen from rat skin and the acid-soluble collagen from guinea pig skin were purified (*8*). The $\alpha 1$, $\alpha 2$, and β_{12} components were isolated by chromatography on carboxymethyl cellulose (*8*). As judged by disc-gel electrophoresis, the β_{11} fraction contained equal amounts of $\alpha 1$ and β_{11}. The $\alpha 2$ component isolated from guinea pig skin collagen was contaminated with β_{12} and was purified by rechromatography.

Rabbit antiserums to collagen were produced by the subcutaneous injection of 20 mg of collagen (dissolved in 4 ml of 0.05 percent acetic acid and emulsified with an equal volume of Freund's complete adjuvant) every 15 days. The rabbits were bled 10 to 14 days after an injection, and the serums obtained from the third bleeding were used.

The microhemagglutination assay (*9*) as modified by Kettman *et al.* (*10*) was performed with tanned, formalin-treated sheep red blood cells sensitized with rat or guinea pig skin collagen or gelatin (0.5 μg/ml). Also, $\alpha 1$ and $\alpha 2$ chains (5.0 μg/ml) and the β_{11} and β_{12} components of collagen (0.5 μg/ml) were coated on tanned, formalin-treated sheep red blood cells (2.5 percent suspension). For measurement of inhibition of hemagglutination, we incubated the stated concentration of collagen component with antiserums, serially diluted twofold, for 1 hour at room temperature before adding antigen-coated cells. When collagen components were added to an unrelated system (tobacco mosaic virus protein and homologous antibodies) there was no inhibition. Immune precipitation of collagen was carried out as described (*7*).

The fact that the antibodies to collagen were directed against determinants that are an integral part of the collagen molecule was shown as follows. (i) Precipitation of collagen with antibodies was abolished by prior incubation of collagen with chromatographically purified collagenase (Worthington grade CLSPA); (ii) the immune precipitate contained hydroxyproline; (iii) the immune precipitate dissolved when incubated with collagenase; and (iv) the expected α or β components, or both, of collagen were detected in the immune precipitate by acrylamide-gel electrophoresis when rat skin collagen or its components were incubated with antiserum to rat skin collagen.

Against homologous antigen, the reciprocal of the hemagglutination titer of antiserum to guinea pig collagen was