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.

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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

Table 1. Hemagglutination titers of antiserums to guinea pig skin collagen reacted with guinea pig skin collagen and with α and β components of guinea pig skin collagen.

Antigen	Titer (reciprocal)
Guinea pig collagen	4096
Guinea pig $\alpha 1$	4–8
Guinea pig $\alpha 2$	2048
Guinea pig $\beta 11$	8
Guinea pig $\beta 12$	1024–2048

4096, and against rat skin collagen it was 8. Likewise, against its homologous antigen, the reciprocal of the hemagglutination titer of antiserum to rat skin collagen was 64,000 and against guinea pig collagen it was 32. These results show great species specificity. Similar results were obtained by the immune precipitin assay.

Three methods were used to determine which components of denatured collagen reacted with antibody to collagen, namely, inhibition of the hemagglutination of collagen-coated red blood cells by prior incubation of the antiserum with the individual polypeptide chains, hemagglutination of polypeptide-coated red blood cells and antiserums to collagen, and precipitin reaction of rat skin collagen with homologous antiserum.

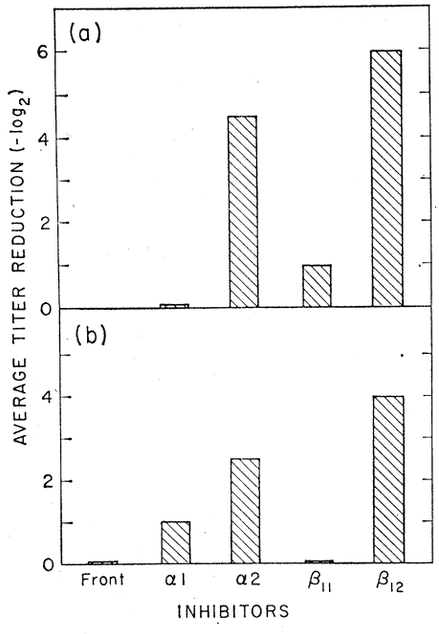

Fig. 1. Inhibition of hemagglutination between collagen and homologous antiserums by polypeptide components of collagen. The fraction labeled "front" represents material not binding to carboxymethyl cellulose (*6*). Diluted serums were incubated for 1 hour at room temperature with homologous collagen components (stock solutions of 10 μg/ml were used before addition of antigen-coated, sheep red blood cells). (a) Antibody to rat skin collagen inhibited with rat collagen components; (b) antiserum to guinea pig skin collagen inhibited with guinea pig collagen components.

The reaction between rat skin collagen and the homologous antibody (Fig. 1a) was inhibited by the $\alpha 2$ component of rat skin collagen whereas $\alpha 1$ had little inhibitory activity. The β_{12} component, which consists of an $\alpha 1$ and an $\alpha 2$ cross-linked by a covalent bond, had somewhat stronger inhibitory activity than $\alpha 2$.

The inhibition of the reaction between guinea pig skin collagen and the homologous antibodies by polypeptide components of the guinea pig skin collagen (Fig. 1b) follows the same pattern as that for rat collagen. Again $\alpha 2$ and β_{12} were the major inhibitors with $\alpha 1$ exhibiting weak inhibitory activity, if any. Both β_{11} and the peak consisting of material not binding to the carboxymethyl cellulose ("front") were devoid of inhibitory activity. Direct hemagglutination of the separated chains of guinea pig collagen with antibodies to guinea pig collagen (Table 1) confirmed the conclusions drawn from the inhibition experiments, namely, that $\alpha 2$ contains the major antigenic determinant or determinants, whereas $\alpha 1$ has essentially no activity. The activity of the β_{12} component is similar to that seen with $\alpha 2$.

Rat skin collagen, the $\alpha 2$ chain, and the β_{12} component precipitate when incubated at various concentrations with antiserum to rat skin collagen (Fig. 2). Some precipitation of $\alpha 1$ and β_{11} also occurred at the concentration of antiserum used. However, the major antigenic determinants are clearly located in $\alpha 2$ and β_{12}.

Treatment of native collagen with various proteases reduces its reaction with antibody (*2–4*). Under our conditions, only the amino terminal peptides would be cleaved from the molecule by the proteases (*4*). For this reason, the activity of various peptides derived by cyanogen bromide cleavage of the isolated $\alpha 1$ and $\alpha 2$ chains of guinea pig skin collagen (*11*) was assayed. The amino terminal peptide of $\alpha 2$, namely, $\alpha 2$-CB1 (10 μg/ml), proved to be a strong inhibitor of the agglutination of red blood cells coated with $\alpha 2$ by rabbit antiserum to guinea pig skin collagen. Little activity was noted in the rest of the digest of $\alpha 2$ or in the digest of the $\alpha 1$ chain, suggesting that a major antigenic determinant of collagen is located in the amino terminal region of the $\alpha 2$ chain.

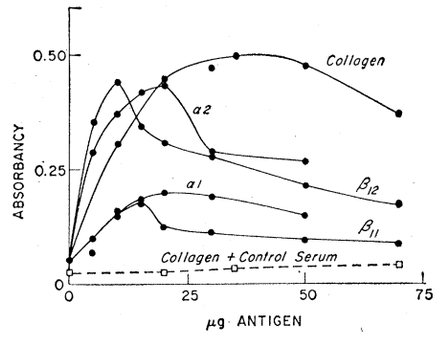

Fig. 2. Precipitin reactions of rat skin collagen or its polypeptide components with antiserum to rat skin collagen. Various amounts of antigen dissolved in phosphate-buffered saline (*p*H 7.2) were reacted with 0.3 ml of serum for 24 to 48 hours at 4°C; final volume was 1.0 ml. Precipitates were washed twice with hemagglutination buffer, dissolved in 1 ml of 0.1*N* NaOH, and 0.3-ml portions were used for protein determination (*13*). Absorbancy was measured at 700 nm.

The $\alpha 2$ chain, which makes up only one-third of the collagen molecule, contains the major antigenic determinant or determinants; as the evidence presented here indicates, the antigenic determinant is located at the amino terminal end of the $\alpha 2$ chain. This region differs in composition from the rest of the chain and is probably not helical. The amino terminal portion of the $\alpha 1$ chains is similar in that, in native collagen molecules, it also is not helical. However, amino acid sequence studies by Piez *et al.* (*12*) indicate that there are interspecies similarities in sequence at the amino terminal region of $\alpha 1$, and disparities in the sequence in the same region of $\alpha 2$.

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Genetic Polymorphism of Tetrazolium Oxidase in Dogs

Abstract. *Two alleles,* To^A *and* To^B, *determine a genetic polymorphism of tetrazolium oxidase in canine erythrocytes. The approximate gene frequencies for most dog breeds are 0.94 for* To^A and *0.06 for* To^B. *The gene frequencies of the German shepherd, 0.8 for* To^A *and 0.2 for* To^B, *differ significantly from those of other breeds. The electrophoretic tetrazolium oxidase isozyme pattern of* Canis latrans *closely resembles the tetrazolium oxidase A pattern of* Canis familiaris.

Brewer demonstrated tetrazolium oxidase in several human tissues and classified the enzyme as an indophenol oxidase (*1*). The exact physiological role of this oxidase is obscure. Its ability to catalyze the transfer of electrons from reduced *p*-nitro blue tetrazolium to oxygen without the presence of a coenzyme suggests a way to detect its site of action after electrophoresis. Unlike the tetrazolium oxidase from human erythrocytes, which presents a common isozyme pattern throughout the species except for rare genetic variants (*1, 2*), the canine oxidase exhibits a genetic polymorphism determined by two alleles, To^A and To^B.

Blood samples were obtained from 189 dogs of various breeds selected at random at a veterinary clinic. Forty-three animals of a beagle colony (*3*), two coyotes (*Canis latrans*), and one coyote-dog hybrid were also tested. In addition, blood samples from 42 offspring of beagles heterozygous at the *To* locus were examined. Fresh blood obtained by venipuncture was absorbed on Whatman 3 MM filter paper (4 by 15 mm). The blood-impregnated paper strips were dried at room temperature and stored at below −20°C. The paper strips were applied to thin-layer starch-gel (1 mm), and electrophoresis (*4*) was performed at 130 volts for 16 hours at 0°C in a gel buffer (*p*H 9.1) containing 0.1*M* tris(hydroxymethyl)aminomethane (tris), $3.25 \times 10^{-3}M$ ethylenediaminetetraacetate (disodium salt), and $1.5 \times 10^{-2}M$ boric acid. The electrode compartments contained 0.3*M* sodium borate buffer, *p*H 8.8. The gels were rinsed for 3 minutes in 0.3*M* sodium borate buffer, *p*H 8.5, and then stained for 2 to 3 hours in daylight in 100 ml of 0.1*M* tris-HCl buffer (*p*H 8.5) containing 15 mg of *p*-nitro blue tetrazolium (Sigma), 15 mg of phenazine methosulfate, and 20 mg of magnesium chloride at 37°C. The stained gels were submerged in a mixture of methanol, acetic acid, and water (5:1:5) for 2 minutes, placed in an aqueous solution of glycerol (15 percent by volume) and acetic acid (2 percent by volume) for 30 minutes, and plasticized at 50°C (*4*).

Canine tetrazolium oxidase, like that from humans and most other mammals, separated on starch gel into many fractions. Isozyme patterns of whole blood and of red cell lysate prepared by lysing erythrocytes that were washed three times with distilled water were identical. The most frequently observed isozyme pattern, type A, consisted of three to four achromatic zones at the anodic side of the gel (Fig. 1). Type A was observed in 78 males and 84 females of the 189 dogs from the clinic series. Type A was also found in 36 (19 male, 17 female) of 43 beagle blood samples. The B isozyme pattern, which resembled the A pattern except for a more cathodic displacement of all tetrazolium oxidase zones, was found only in one dog, a wirehaired fox terrier. The remaining blood samples from the clinic series (14 male and 12 female) and three male and four female samples from the beagle colony exhibited a more complex zonal pattern AB (Fig. 1). The electrophoretically fastest and slowest zones of the AB pattern resembled in mobility the A and B fractions, respectively. In between the A and B regions and partially superimposed on them lay additional achromatic zones. The AB pattern suggested that such individuals synthesized both the A and B tetrazolium oxidase isozyme series and also a series of molecular hybrid isozymes of intermediate electrophoretic mobility, altogether probably as many as 12 isozymes of the oxidase. The canine heterozygous tetrazolium oxidase pattern appeared to be analogous to the oxidase isozyme pattern observed in human heterozygotes (*2*). The oxidase isozyme pattern of the two coyotes and one hybrid animal were electrophoretically indistinguishable from the canine oxidase type A.

The zymogram patterns suggested the presence of two codominant alleles at the canine *To* locus. The codominant transmission of the *To* character was confirmed by nine backcross matings which produced 20 A and 22 AB offspring. This result agrees with the expected ratio of 1:1 (Table 1). Transmission of the B gene from father to son was observed four times, indicating

Table 1. The *To* backcross matings of beagles.

Litters (No.)	Whelps (No.)*			
	Male AA	Female AA	Male AB	Female AB
	Sires AA × dams AB			
5	3	5	3	10
	Sires AB × dams AA			
4	6	6	4	5

* Survivors of litters 4 to 10 months old.

Table 2. The *To* gene frequencies in the dog.

Breed	Dogs (No.)	Heterozygotes (No.)	Allele A	Allele B
Beagle colony, a	43	7	0.919	0.081
German shepherd, b	20	8	0.800	0.200
Terriers, c	14*	1	0.893	0.107
Cocker spaniel, d	13	1	0.962	0.038
Poodle, e	19	1	0.974	0.026
Dachshund, f	21	1	0.976	0.024
Miscellaneous, g	94	12	0.936	0.064
Pooled, c–g	161	16	0.944	0.056

* Includes one B homozygote.