

IDIOTYPIC CROSS-REACTIVITY BETWEEN ANTIBODIES OF DIFFERENT SPECIFICITIES*

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Idiotypic cross-reactivity has been demonstrated between a number of closely related antibody populations. Williams et al. (1) were able to demonstrate idiotypic cross-reactivity between 10 human cold agglutinins of the IgM class. It has also been shown that this phenomenon exists among human IgM proteins with anti-human IgG activity (2-4). Idiotypic cross-reactivity has been demonstrated using inbred strains of mice (5-10), and rabbits (11), as well as some interstrain cross-reactions in mice (12, 13) and goats (14). Interspecies cross-reactivity has been demonstrated between goats and sheep (15). All of these have used antibodies of the same specificity to elicit the anti-idiotypic response and study cross-reactions. When dealing with antibody populations which possess different specificities, cross-idiotypic specificity is not generally noted.

A number of studies have demonstrated that antibodies of different specificities do not share common idiotypes (16-19). However, Oudin and Cazenave (20) have reported idiotypic cross-reactions in rabbit anti-ovalbumin populations of different specificities. In that study, a population of gamma globulin molecules was also found that lacked binding activity for ovalbumin but also exhibited the cross-reacting idio-*type*.

The system that is dealt with in this paper is one in which the antibodies produced are directed toward the antigen, human sickle cell hemoglobin (HbS).¹ Anti-idiotypic serum is raised, in rabbits, against a site-specific antibody isolated from this antiserum, anti-Val (15). This antibody population is specific for the β^6 -position of HbS and will not react with normal human hemoglobin. Cross-idiotypic reactions are studied using this antiserum and different antibody populations from the anti-HbS serum along with other antibody populations. Cross-idiotypic specificity can be demonstrated in radioimmunoassay experiments between antibody populations of different specificities. The further fractionation of these antibodies suggests that in one case the antibodies which contain a cross-reacting idio-*type*, although they have a different specificity, are directed towards the same antigenic site on the hemoglobin molecule as in the anti-Val antibody population. However, this does not seem to be a general property of such idiotypically cross-reacting antibody populations.

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¹ Abbreviations used in this paper: HbA, human adult hemoglobin; HbC, human hemoglobin C (β^6 Glu-Lys); HbS, sickle cell hemoglobin; RIA, radioimmunoassay.

Materials and Methods

Hemoglobin. HbS and human hemoglobin C (β^6 -Glu-Lys) (HbC) were obtained from patients who were heterozygous for these hemoglobins. The HbS was separated from human adult hemoglobin (HbA₁) as described by Karol et al. (15). HbC was purified as described by Dozy et al. (21) with some modification. A 20-cm³ packed column with dimensions (1 × 15 cm) was used. The column was equilibrated using 0.05 M phosphate-0.03 M citrate buffer, pH 7.9. The hemoglobin mixture was loaded onto the column and a linear gradient was started using the original buffer and 0.05 M phosphate-0.03 M citrate buffer, pH 7.2. The same elution profile was obtained as that of Dozy et al. (21).

The α - and β - chains of HbA₁ were prepared as described by Bucci and Fronticelli (22).

Anti-HbS Sera. Goats and sheep were immunized with HbS as previously described by Noble et al. (23).

Isolation of the Total Anti-HbS Antibodies which Cross-React with HbA (anti-HbA^{cr}). HbA₁ was conjugated to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) as instructed by the manufacturer. The purified anti-HbS antibody populations were isolated as described by Karol et al. (15). The antibody population, which was eluted from the HbA₁-Sepharose 4B column with 1 M acetic acid, 0.5 M sodium chloride, was called anti-HbA cross-reacting (anti-HbA^{cr}).

Isolation of Anti-Val. The antibody population that binds HbS and not HbA₁, anti-Val, was isolated as described by Karol et al. (15).

Isolation of Precipitating and Nonprecipitating Anti-HbA^{cr} Populations. A specific antigen-antibody complex was formed at equivalence as determined by quantitative precipitin data, with the anti-HbS serum and HbA₁. This precipitate was washed with normal saline and suspended in 0.2 M sodium acetate buffer, pH 4.3. The pH was adjusted to 4.0, and pepsin was added in the amount of 1% of the total protein weight. The solution was made 0.01 in sodium azide to prevent bacterial growth. The digestion was allowed to proceed for 24 h at 37°C. At the end of this time, a dark brown precipitate formed which was spun down and discarded. This precipitate contains the heme group which has been released because the globin chains are digested into small fragments by the pepsin. The colorless supernate was carefully brought to pH 8.0 with 1.0 N sodium hydroxide, and then dialyzed against 100 vol of 0.05 M phosphate borate pH 8.0 buffer. This procedure yields (Fab')₂ (dimers) of the precipitating portion of the anti-HbA cross-reacting material.

The nonprecipitating fraction of the anti-HbA^{cr} material is isolated by using the anti-HbS serum which has had all of the precipitating anti-HbA^{cr} antibodies removed by the procedure described above and passing it through a column of HbA₁-Sepharose 4B according to the methods previously described.

Fractionation of Anti-HbA Cross-Reacting Population (anti-HbA^{cr}). The population of antibodies termed anti-HbA cross-reacting was further fractionated. The fractions that would bind to one or the other of the isolated subunits were obtained by passage of the anti-HbS serum through a Sepharose 4B column to which the hemoglobin chain (α or β) was bound. The antibodies were isolated by elution with 1 M acetic acid, 0.5 M sodium chloride, as previously described. These two antibody populations will be referred to as anti- α and anti- β^A portions of the anti-HbA^{cr} material.

The anti- β^A -chain population was further fractionated by passage through a HbC-Sepharose 4B column. The antibody population that will not bind to the HbC, and passes through the column, is referred to as anti- β^6 specific, because that is the site at which HbC differs from HbA. The antibody population that binds to the HbC-Sepharose 4B is referred to as the anti-HbC^{cr} fraction.

The anti-HbA^{cr} population was put through a HbA-Sepharose 4B column a second time to detect any antibody population that will not bind. The protein that does not bind to the second column is called the nonbinding portion of anti-HbA^{cr}; the fraction that binds is called anti-HbA^{cr2}. These two fractions are used in radioimmunoassay (RIA) competition experiments.

Isolation of Goat Anti-HbA and Goat Anti-Cytochrome C. Goat serum containing antibodies directed towards either HbA₁ or human cytochrome c was passed through a Sepharose 4B column to which the respective antigen had been bound. The same elution procedure was followed as described above.

Normal Goat Gamma Globulin and Normal Sheep Gamma Globulin. These are obtained commer-

cially from either Schwarz/Mann Div., Bectin, Dickinson, & Co., Orangeburg, N. Y. or U. S. Biochemical Corp., Cleveland, Ohio.

Fluorescence Quenching. Fluorescence quenching experiments were performed by the method of Noble et al. (24). The antibody populations were digested with pepsin in the presence of 0.1 M cysteine and alkylated with iodoacetamide as described by Tan-Wilson et al. (25). The concentrations of antigen and Fab were determined as described by Tan-Wilson et al. (25).

Anti-Idiotypic Antisera. Anti-idiotypic antisera directed towards goat anti-sheep anti-Val were obtained as was previously described by Karol et al. (15).

RIA Experiments. These RIA experiments were performed as previously described by Karol et al. (15).

Results

Idiotypic Cross-Reactivity with Anti-HbA^{cr} Population. The anti-idiotypic serum used (Ra 326) was obtained by immunization of a rabbit with the site-specific antibody population, goat 6 anti-Val (15). This antiserum does not cross-react with normal goat gamma globulin or goat antibodies directed towards normal human hemoglobin (HbA₁), Fig. 1. However, when the fraction of the goat 6 anti-HbS antibody population that binds to HbA (anti-HbA^{cr}) was used, very strong inhibition was obtained. The majority of the anti-idiotypic reaction is inhibited by this population of antibodies which has a different specificity than the antibody population used to elicit the anti-idiotypic response.

Similar results have been obtained using rabbit antibodies directed toward the idio type present on sheep anti-Val antibodies. The anti-HbA^{cr} antibody fraction of the total sheep anti-HbS response, inhibits the reaction of rabbit anti-idiotypic serum with the homologous sheep anti-Val, Fig. 2. Cross-reacting idiotypic determinants are also present in a heterologous sheep (no. 27) anti-HbA^{cr}, goat 6 anti-HbA^{cr}, and goat anti-HbA antibodies from an anti-HbA serum.

Specificity of Anti-HbA^{cr} Antibody Populations. To test for the possibility that within the anti-HbA^{cr} population there exists a subpopulation of antibodies with a much higher affinity for HbS than HbA, both antigens were used in fluorescence quenching and RIA experiments. The results of these experiments are shown in Figs. 3 and 4. The differences observed between the two antigens are very slight and within the error of the measurements. Therefore, there does not appear to exist a major antibody fraction within the anti-HbA^{cr} population, that is specific for HbS.

To further insure that there did not exist a population of antibody within the anti-HbA^{cr} population that is actually specific for HbS, a known quantity of anti-HbA^{cr} antibody is passed through a HbA-Sepharose 4B column. It was found that all of the active antibodies bound to the HbA-Sepharose 4B column. Any protein that did not bind to the HbA-Sepharose 4B, as determined by OD₂₈₀, did not bind to HbS-Sepharose 4B either. It is assumed that this material represented denatured antibody. The material that did not bind to the HbA-Sepharose 4B column was used in a RIA-competition experiment to determine if it was actually anti-Val. To insure that a small contamination of anti-Val could be detected by this method, anti-Val was added to goat anti-HbA so that it represented 2% of the total protein and the same procedure used. The results are shown in Fig. 5. The nonbinding material from the anti-HbA—anti-Val mixture inhibited the anti-idiotypic reaction quite strongly. However, it did not show an identical inhibition pattern as the cold anti-Val. The reason for this is that the actual concentration of anti-Val in this fraction is difficult to determine because some denatured anti-HbA material was probably present. This

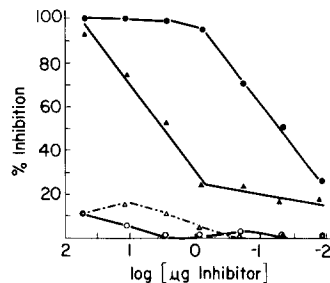


FIG. 1. Inhibition of binding of ^{125}I -labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: ●, goat 6 anti-Val; ▲, goat 6 anti-HbA^{cr}; ○, normal goat gamma globulin; △, goat 4 anti-HbA₁.

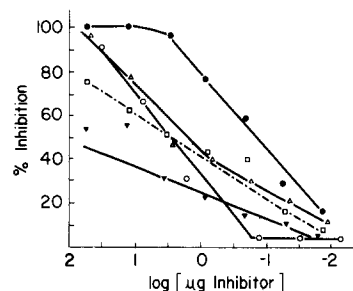


FIG. 2. Inhibition of binding of ^{125}I -labeled sheep 26 anti-Val to Ra 585 anti-idiotypic serum by: ●, sheep 26 anti-Val; ○, sheep 26 anti-HbA^{cr}; △, sheep 27 anti-HbA^{cr}; □, goat 6 anti-HbA^{cr}; ▼, goat 4 anti-HbA₁.

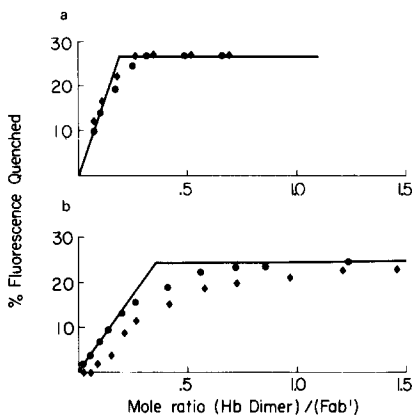


FIG. 3. Fluorescence quenching titrations of (a) goat 6 anti-HbA^{cr} and (b) sheep 26 anti-HbA^{cr} with HbS, ●, and HbA₁, ◆.

is indicated by the fact that more material was found in this nonbinding fraction than the original amount of anti-Val added to the mixture. Therefore, the existence of something else in this fraction is almost certain. The nonbinding material from the anti-HbA^{cr} fraction showed little cross-reactivity. The cross-reactivity of the material that bound to the HbA-Sepharose 4B column the second time was not eliminated. The change in this level of inhibition is probably due to two exposures of these antibodies to acid elution. The actual concentration of functional antibody molecules in this fraction has not been determined.

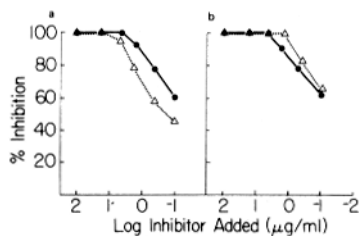


FIG. 4. Inhibition of the binding of ^{131}I -labeled HbS to (a) goat 6 anti-HbA^{cr} and (b) sheep 26 anti-HbA^{cr} by: ●, HbS; and HbA₁, Δ.

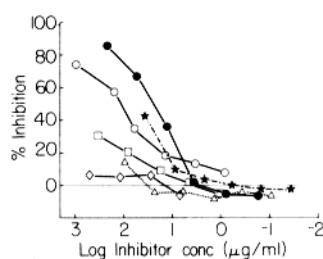


FIG. 5. Inhibition of the binding of ^{131}I -labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: ●, goat 6 anti-Val; ○, goat 6 anti-HbA^{cr}; ★, the non-HbA-binding eluate from anti-Val seeded goat 4 anti-HbA₁; Δ, non-HbA-binding eluate from goat 6 anti-HbA^{cr}; ◇, goat 4 anti-HbA₁; □, goat 6 anti-HbA^{cr2}.

Idiotypic Cross-Reactivity of the Fractions of Goat 6 Anti-HbA^{cr} Population. An attempt was made to fractionate the goat 6 anti-HbA^{cr} antibody population. Precipitating and nonprecipitating fractions of the anti-HbA^{cr} population were isolated, as described in Methods, to determine if the idiotype would be present in one of these fractions and not the other. As is demonstrated by an RIA competition experiment with Ra 326, the cross-reactive idiotype was present in both the precipitating and nonprecipitating fraction, Fig. 6.

Another method of fractionating this antibody population was used. The populations of antibodies which are specific for the isolated subunits of HbA, were obtained as described in Materials and Methods. These subpopulations, which will be referred to as anti- α and anti- β^A (although it must be emphasized that these are isolated from antiserum which is prepared using HbS as an antigen), are used in the same type of ORIA competition experiments with Ra 326 serum as previously described. The results of such an experiment are shown in Fig. 7. The antibody population directed towards the α -chain of HbA did not demonstrate idiotypic cross-reactivity, whereas the antibody population directed towards the β -chain of HbA exhibited strong idiotypic cross-reactivity. The level of inhibition was not identical with that seen for total anti-HbA^{cr} response. The reason for this is not yet clear but is probably a result of the second exposure of these antibodies to acid elution.

The antibody population directed towards the β -chain of HbA was further fractionated. Another mutant hemoglobin, HbC (β^6 -lysine) was used. The anti- β^A antibodies were passed through a HbC-Sepharose 4B column. A fraction failed to bind. This antibody population must be directed toward the region of the β^6 residue and was labeled anti- β^6 specific, whereas the antibody population which bound to the HbC-Sepharose column was called anti-HbC^{cr}. The results of the RIA-competition

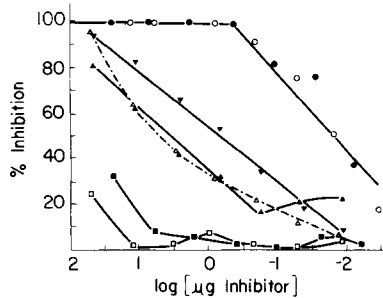


FIG. 6. Inhibition of the binding of ¹³¹I-labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: ●, goat 6 anti-Val; ○, goat 6 anti-Val modified with cold iodine; ▲, goat 6 anti-HbA^{cr}; ▼, precipitating goat 6 anti-HbA^{cr}; △, nonprecipitating goat 6 anti-HbA^{cr}; □, normal goat gamma globulin; ■, guinea pig anti-Val.

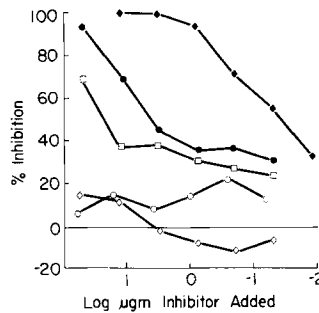


FIG. 7. Inhibition of the binding of the ¹³¹I-labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: ◆, goat 6 anti-Val; ●, goat 6 anti-HbA^{cr}; ○, goat 6 anti-α chain; □, goat 6 anti-β^A chain; ◇, goat 4 anti-HbA.

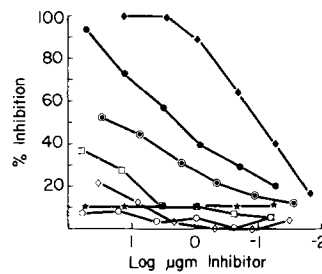


FIG. 8. Inhibition of the binding of ¹³¹I-labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: ◆, goat 6 anti-Val; ●, goat 6 anti-HbA^{cr}; ○, goat 6 anti-α chain; □, goat 6 anti-β^A chain; ◇, goat 6 anti-HbC^{cr}; ⊙, goat 6 anti-β⁶ specific; ★, normal goat gamma globulin.

experiment using these antibody populations are shown in Fig. 8. The anti-β⁶-specific population exhibited strong idiotypic cross-reactivity, whereas the antibody populations directed toward the other determinants on HbA did not (anti-HbC^{cr} population). The level of inhibition of the anti-β⁶-specific population was higher than that of the total anti-β. However, these data cannot be dealt with quantitatively because these isolated goat antibody populations are very unstable. Therefore, the estimated concentrations of the inhibitors may be in error as no determination of the amount of denatured material was carried out.

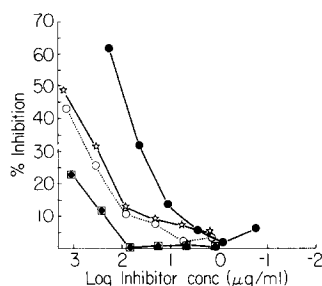


FIG. 9. Inhibition of the binding of ^{131}I -labeled sheep 26 anti-Val to Ra 585 anti-idiotypic serum by: ●, sheep 26 anti-Val; ☆, sheep 26 anti-HbA^{cr}; ○, sheep 26 anti- α chain; ◻ sheep 26 anti- β^A chain.

Idiotypic Cross-Reactivity of the Fractions of Sheep Anti-HbA^{cr} Population. The same fractionation procedure used for the goat antibody populations was also used for the sheep antibodies. These fractions: anti-HbA^{cr}, anti- α , anti- β^A , were used in RIA-competition experiments. The results using the subpopulations of the sheep anti-HbA^{cr} fraction are quite different from that seen with the rabbit anti-goat idiotype system (Fig. 9). As shown, both the anti- α and anti- β^A subpopulations effectively cross-react with the anti-idiotypic serum. In fact, the sheep anti- α demonstrates higher inhibition levels than the sheep anti- β^A . Similar results are obtained using the fractions of goat anti-HbA^{cr} with this anti-idiotypic serum.

Discussion

The occurrence of idiotypic cross-reactivity between anti-Val antibodies, which bind only HbS, and anti-HbA^{cr} antibodies, which bind both HbS and HbA₁, has been demonstrated in RIA-competition experiments.

The possibility that the cross-idiotypic specificity of the anti-HbA^{cr} population is actually due to a contamination of anti-Val antibodies was considered. It can be calculated that a 2% contamination of anti-Val in the anti-HbA^{cr} fraction would give the results obtained. No significant differences were observed in the ability of anti-HbA^{cr} Fab' fragments to bind HbA₁ and HbS in either RIA or fluorescence quenching experiments. However, a small contamination may not have been detectable. An attempt to isolate the hypothetical contaminant fraction by passage through a second HbA-Sepharose column and detection by an RIA-competition experiment was unsuccessful. In this experiment, to insure that a 2% contamination would be detectable, a goat anti-HbA antibody population was seeded with anti-Val so that there was a 2% contamination. The nonbinding eluant from the seeded anti-HbA mixture inhibited the anti-idiotypic reaction quite strongly. Therefore, it is assumed that the cross-idiotypic specificity in the anti-HbA^{cr} population is not due solely to anti-Val contamination. Further evidence to support the idea that this cross-reactivity is not due to anti-Val contamination is the fact that there is no partitioning of the cross-reactive population in the precipitating and nonprecipitating fractions of the anti-HbA^{cr} antibodies. Anti-Val would not be included in the precipitating portion of anti-HbA^{cr}.

The fractionation of the goat anti-HbA^{cr} population clearly establishes that the cross-idiotypic specificity can be isolated in an antibody fraction specific for the β^6 region of the hemoglobin molecule. This antibody population differs from anti-Val in its specificity towards antigen, yet it contains a cross-reactive idiotype.

A plausible hypothesis about the amino acid residues in the anti-Val and the anti-HbA^{cr} molecules which are responsible for the observed antibody and idiotypic specificities is the following. Anti-Val and anti-HbA^{cr} share a number of amino acid residues complementary to a number of amino acids on HbS, among which a key residue is the β^6 -position. Most of the amino acid side chains of the anti-Val and anti-HbA^{cr} molecules are identical, but at least one side chain is different which is responsible for the difference in the observed binding specificity. The shared residues are responsible for the strong idiotypic cross-reactivity.

The fractions of sheep anti-HbA^{cr} react quite differently from the corresponding goat fractions. The anti- α portion of the anti-HbA^{cr} response contains a cross-reactive idio type as well as the anti- β population. Goat antibodies directed toward HbA also cross-react with this anti-idiotypic serum, whereas they do not cross-react with the rabbit anti-goat idio type. It should be noted that this is an interspecies cross-reaction. This fact also eliminates the possibility of this cross-reaction being due solely to anti-Val contamination, because there is no anti-Val present in the goat anti-HbA population. Sheep anti-HbA antibodies were not available for testing. It is possible that the sheep and goat differ in their response to the antigen HbS so that sheep produce an antibody population specific for the β^6 -region of HbS which contains an idio type which is similar to that of a population of antibodies that are directed towards a site of the α -chain of the hemoglobin molecule. This site (or sites) may be similar to the β^6 -region of HbS but there is no evidence for or against this possibility. The regions on the human α -chain that are antigenic in the sheep and are responsible for this cross-reactivity have not yet been identified.

We have confirmed the observation of Oudin and Cazenave (20) that idiotypic cross-reactivity is not a sufficient condition for identity of specificity. Such cross-reactivity has now been observed between a number of antibody populations with clear differences in their immunological specificities. Such cross-reacting populations may have closely related specificities, as in the case of the anti-Val and anti- β^6 -specific antibodies, but this apparently is not necessary. It is not difficult to imagine how the substitution of a small number of amino residues in the sequence of the variable regions of an antibody molecule could cause a major change in specificity although not eliminating the idiotypic relationship to the original antibody molecule. A minority of the anti-goat anti-Val and anti-sheep anti-Val antibodies used in this study are directed toward the site of antigen (HbS) binding (14, 15), clearly permitting a dissociation between idio type and specificity. We conclude that, more than being imaginable, idiotypic cross-reactivity between antibodies of different specificities actually occurs.

Summary

Cross-idiotypic specificity has been demonstrated between antibody populations of different specificities using antibodies directed toward human sickle cell hemoglobin (HbS). A site-specific antibody directed toward the β^6 -position of HbS, anti-Val, was used to elicit an anti-idiotypic response in rabbits. Using this anti-idiotypic serum, idiotypic cross-reactivity was demonstrated between antibody populations that bind to human adult hemoglobin (HbA). It was demonstrated that in the case of the goat antibodies, these idiotypically cross-reacting antibodies are directed towards the β^6 -position of the hemoglobin molecule. However, they differ in their specificity, binding to this site on HbA, whereas anti-Val binds only to HbS.

The sheep antibody populations directed toward HbS differ qualitatively from those of the goat. Using rabbit anti-idiotypic serum specific for sheep anti-Val, cross-reactivity could be demonstrated with antibodies directed toward the α -chain of the hemoglobin molecule, as well as the β -chain. There was also a low level of cross-reactivity with antibodies from a goat immunized with HbA.

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