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THE PRODUCTION OF RH SUB 0 (D) NEGATIVE ERYTHROCYTES  
(U) NEW YORK BLOOD CENTER N Y J GOLDSTEIN 18 JUL 86  
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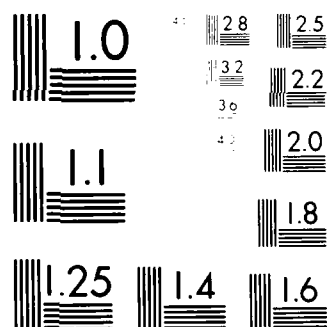
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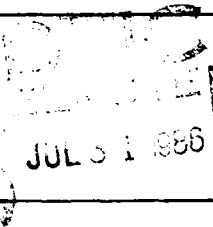
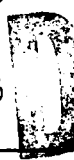
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  Attempts are being made to modify or destroy the Rh D antigen under conditions which will produce Rh negative erythrocytes of transfusable quality. One of the approaches being taken involves the isolation and biochemical characterization of the D antigen in order to gather information which will help formulate ways of modifying its expression. To this end we have progressed toward establishing a 28-32 Kd membrane protein as the primary D antigen. This is evidenced by its ability under certain conditions to			

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20. Abstract

induce antibody toward Rh positive cells and when attached to plates to yield a positive reaction with human anti-D in the ELISA assay.

Our overall goal is to produce Rh D negative erythrocytes which can be used in transfusion therapy. Last year we reported our initial attempts to develop procedures for the removal or inactivation of the D antigen and the determination of its biochemical make-up. This year we have focused on the isolation and biochemical characterization of the Rh D antigen. Aside from the intrinsic value of this approach since nothing is known about D antigenic structure, the information obtained will help formulate methods for modifying its expression and thus mesh very well with our other approaches directed toward nullifying the D antigen on the surface of the red cell.

Many of the techniques relevant to accomplishing this goal have now been established in our laboratory. We have optimized the conditions for the isolation of the D antigen as part of an immune complex. Briefly, this approach involves reacting Rh positive erythrocytes of phenotype cDE/cDE which contain approximately 40,000 D antigenic sites/cell with human serum containing high titer IgG anti D. The cells are then lysed, membranes which contain antibody attached to the D antigen are isolated and then solubilized using either the detergent Triton X 100 at a concentration of 1% in phosphate buffered saline pH 7.4 or 0.1% trifluoroacetic acid (TFA) in water. The immune complex consisting primarily of D antigen bound to anti D is then isolated by absorption to either protein A Sepharose or anti IgG Sepharose beads and eluted at 37° C with 1% sodium dodecyl sulfate. The D antigen is currently being separated from the anti D of the immune complex by SDS-PAGE and believed to coincide with the 28-32 Kd band obtained from such fractionations.

Several approaches are being taken to establish that the 28-32 Kd protein is the D antigen. One involves the Western immunoblotting procedure i.e. transfer of the 28-32 Kd protein region from SDS-PAGE to nitrocellulose paper, treatment of the transferred protein band with high titer human IgG containing anti D, washing off the unreacted antiserum and then reacting with peroxidase labeled anti human IgG. Following a second washing a peroxidase sensitive dye-substrate serving as the indicator is added with a positive reaction yielding a colored product. Initial results were negative which is not unexpected since many antibodies that recognize native antigens do not react with their SDS denatured counterparts and this human anti D polyclonal antibody reacts quite strongly with the native D antigen on the cell surface. It will be repeated using larger amounts of the 28-32 Kd protein band since we are now preparing it in an ongoing basis.

This material is also being injected into rabbits and mice in an attempt to produce antibodies that will react with it and D+ erythrocytes thus establishing the 28-32 Kd protein as the D antigen. Only a very weak response, thus far, toward D+ erythrocytes has been obtained with rabbits. Mice on the other hand, produce significant amounts of antibody under certain conditions. Their immunization is carried out in the following way. Following electrophoresis of the immune complex the 28-32 Kd region is cut out, minced, homogenized and then lyophilized. The dried material is taken up in a small volume of PBS and 100  $\mu$ l (5 $\mu$ g of protein) injected intrasplenically into approximately six week old female Balb/c mice. The next injection was given subcutaneously three days later. Thereafter injections of the same amount of material were made every other week for one and one-half months. Blood samples were collected ten days after the first injection, then every other week and agglutination titers measured against Rh D positive and negative cells using the Coombs' (anti IgG) procedure. To date, four such studies have been performed, in two of which treated mice produced antibodies having serial dilution titers of 1:256 and 1:128 when tested against Rh D positive cells with negligible reaction against Rh negative cells. These antibody titers are transitory however, reaching a peak after the fourth injection and then losing one-half of their activity every two weeks. We are currently exploring ways to obtain higher titer and longer lasting production of antibodies since these antisera do not as yet yield positive Western blots against the 28-32 Kd region. When appropriate titers are obtained, we

plan in collaboration with Dr. Jay Valinsky to prepare monoclonal antibodies to this protein fraction.

Recent experiments involving another approach namely the ELISA assay procedure show that when 28-32 Kd protein is attached to plates it yields a positive reaction with human anti D antisera. These results provide further evidence for the D antigenic structure being associated with the 28-32 Kd protein.

Amino acid sequencing and amino terminal analysis is also underway. In order to perform these studies we have succeeded in adapting the recently reported electroblotting procedure for transferring the 28-32 Kd protein region from SDS-PAGE gels to derivatized glass filters. These filters can then be used directly in gas phase sequencers which employ the Edman degradation procedure. Using this technique amino acid sequencing can be performed at the picomole level which in our case means 20-100 picomoles of the 28-32 Kd protein region. This work is being done in collaboration with Dr. Steven Kent of the California Institute of Technology who has developed a great many of these micro protein analytical procedures. The results of these studies will establish the chemical identity of the 28-32 Kd protein region. Our first analysis revealed the presence of the amino acids lysine, tyrosine and proline at positions three, four and five of the peptide chain. The reason for obtaining only a partial sequence was believed to be due to the presence of SDS, which was used in our isolation procedure, interfering in the sequencing. As mentioned earlier we have now developed a procedure which employs TFA instead of SDS for isolation of 28-32 Kd protein containing immune complex. When such SDS-PAGE purified material was recently analyzed neither an amino terminal amino acid nor any amino acid sequence was detected suggesting that the 28-32 Kd protein contains a blocked amino terminus. Should this be the case limited digests of this protein will be prepared using both cyanogen bromide and various proteases. The peptides will be separated by high performance liquid chromatography (HPLC) and then sequenced in order to determine the structure of the 28-32 Kd protein. As a corollary to this approach attempts are also being made to separate the putative D antigen from the immune complex other than through the use of SDS-PAGE. A variety of chromatographic procedures both classical and HPLC are being explored. Once such a method is developed it would allow us to obtain relatively larger amounts of the 28-32 Kd protein, serve as a further check on its purity and thus help establish the chemical identity of the putative D antigen.

#### Goals for 1986-87

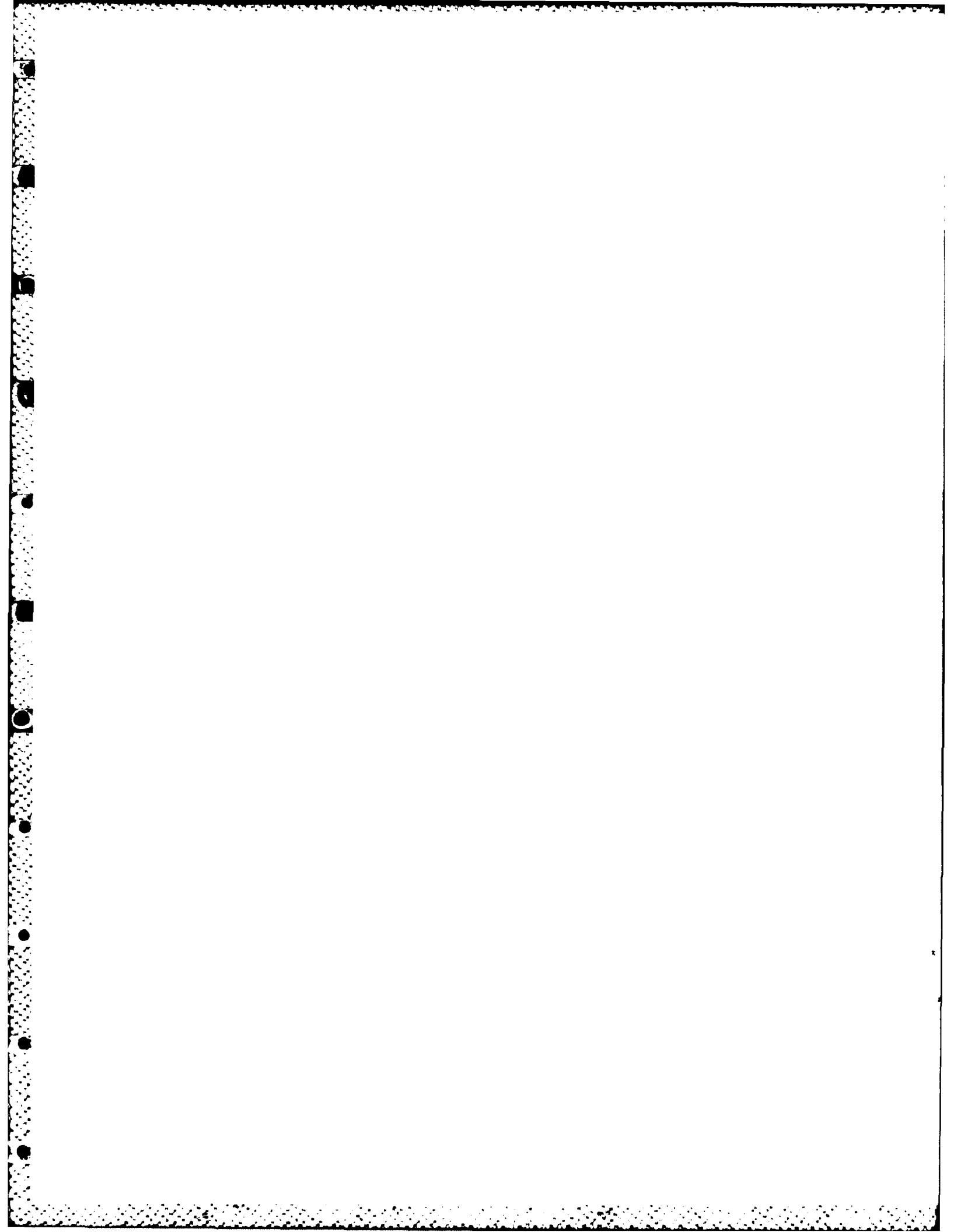
We will continue our biochemical and immunological characterization of the 28-32 Kd protein in order to confirm its identity as the primary D antigen.

We also will continue our measurement of the effects of various membrane perturbants and enzymes upon the expression of D antigenicity on the cell surface.

#### Publications

"Isolation of a Red Cell Membrane Component Expressing Rh D Antigenicity." K. Suyama and J. Goldstein. *Am. Soc. Cell Biol.*, Washington, D.C. Dec 7-11, 1986.

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