The study suggests that promethazine is a potent stimulator or inducer of enzyme and protein synthesis and the benefit of promethazine in the treatment of Rh erythroblastosis may relate to enhancement of bilirubin disposal mechanisms.

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Antibody-dependent Cell-mediated Destruction of Human Erythrocytes Sensitized in ABO and Rhesus Fetal-Maternal Incompatibilities

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Extract

Antibody-dependent cell-mediated cytotoxicity (ADCMC) was measured in a ^{51}Cr-release assay with antibody-sensitized human red blood cells (RBC) as target cells and nonimmune isologous or autologous mononuclear peripheral blood leukocytes (MPBL) as effector cells. ADCMC was readily demonstrable within 6 hr and was independent of exogenous complement (C'). The specificity of the ADCMC reaction was determined by the antiserum employed. Anti-A, anti-B, anti-D, and anti-c sera were all active in ADCMC; the specific lysis was between 35 and 50% with antiserum dilution of 1/1,000, using 10^5 target cells and 10^6 MPBL. Anti-C, anti-E, and anti-e sera had no detectable activity. Sephadex column separation suggested that this activity was by antibodies of the IgG class.

Cord RBC from infants with RhD fetal-maternal incompatibility were lysed by autologous or isologous MPBL in the absence of exogenous antiserum. ADCMC was between 22% and 42% in cases with high direct Coombs' test, but was negative with low direct Coombs' test as in five cases of ABO incompatibility.

Speculation

In hemolytic disease of the newborn, the precise mechanisms involved in the destruction of fetal erythrocytes sensitized with maternal antibodies are not well known. The experiments described in this report raise the possibility that ADCMC could be operative *in vivo*.

The precise events leading to the destruction of fetal erythrocytes in hemolytic disease of the newborn are not known. There is evidence that fetal erythrocytes coated with maternal IgG antibodies can be lysed by cell-mediated mechanisms (3–5). In this context, the potential interactions between mononuclear cells, red cells, and IgG in man have been extensively investigated (1, 2, 10–12). Recent results by Holm and his colleagues (7–9) have demonstrated ADCMC against human RBC pretreated with isologous antisera to blood group antigens, mediated by nonimmune human MPBL. Using erythrocytes as target cells, the effector population for ADCMC among mononuclear cells appears to be monocytes. Antibody-dependent cytotoxic lymphoid cells (K cells) are not efficient killers of human erythrocytes, although they are extremely active against sensitized nucleated target cells (13, 15).

In this paper we report a study of ADCMC in fetal-maternal incompatibilities.

MATERIALS AND METHODS

MEDIUM

The medium used throughout this study was Dulbecco's modified Eagle's medium (16) supplemented with 5% heat-inactivated (56° for 45 min) fetal calf serum, penicillin, streptomycin, and bicarbonate as buffer.

TARGET CELLS

Fresh human RBC in ACD were genotyped for ABO and Rh antigens (17). After two washings in Hanks' balanced salt solution, 20–30 $\times$ 10^6 RBC in 0.2 ml buffer (0.8 g NaCl, 0.038 g KCl, 0.01 Na_2PO_4, and 0.3 g Tris, 100 ml, adjusted to pH 7.40 with HCl) were labeled for 30 min with 200 μCi ^{51}Cr (Na_2CrO_4, 1 mCi/ml, specific activity 200 μCi/mg Cr or more) (18). The target cells were then washed twice with Hanks' solution and resuspended in medium at a concentration of 0.5 $\times$ 10^6/ml.

When RBC from cord blood were used as target cells, they were washed four times with 0.9 M NaCl at 37° and then once in Hanks' solution before labeling with ^{51}Cr.

ANTISERA

Antisera to Rh System. Both maternal and commercially available human hyperimmune antisera were used. The commercial preprations included: anti-D serum (DADE (19) catalog no. B 4665, lot D-616 EC and D-625 GW), anti-c serum (DADE (19) catalog no. B 4695), anti-C serum (DADE (19) catalog no. B 4685), anti-e serum (DADE (19) catalog no. B 4716) and anti-E serum (DADE (19) catalog no. B 4705). These antisera had no C′-dependent lytic activity. All antisera were inactivated by heating at 56° for 45 min. Anti-D sera had hemagglutination titers (indirect Coombs' test) of 1:524,288 or more. One lot (D-616 EC) contained 6.3 IU/ml IgG and 12.0 IU/ml IgM, measured by a radial diffusion technique (6, 14).

Anti-A and Anti-B Sera. Both maternal and commercially available human hyperimmune antisera were used. Commercial preparations came from DADE (19): anti-A serum (catalog no. B 4627-1, lot A-478 XX), anti-B serum (catalog no. B 4627-2, lot B-590 WY). These antisera had C-dependent hemolytic activity for RBC of the relevant blood group but not after inactivation at 56° for 45 min. The hemagglutination titer of each antiserum was greater than 1:524,288.

EFFECTOR CELLS

Human MPBL of group O Rh^- donors were separated from heparinized whole blood by Ficoll-Isopaque gradient centrifugation (d = 1.077, 650 $\times$ g, 20 min, 20°). The white cell layer at the interface was collected, washed twice in Ca^{++}- and Mg^{++}-free Hanks' solution, and centrifuged at 150 $\times$ g to remove thrombocytes. After erythrocyte lysis by incubation for 5 min in 0.88% NH_4Cl in Tris buffer, pH 7.6, the cells were washed in Hanks' solution and resuspended in medium at 2 $\times$ 10^6 cells/ml. Differential counts of cells on cytocentrifuge smears stained with May-Grünwald Giemsa revealed that the MPBL effector cell population consisted of approximately 80–85% lymphocytes, 15–20% monocytes, and 0–3% polymorphonuclear leukocytes. More than 95% of the cells were viable as assessed by trypan blue dye (0.05%) exclusion Cord blood MPBL were prepared by the same procedure.

CYTOTOXICITY ASSAY

The following standard experimental conditions were used for ADCMC tests, unless otherwise stated. The effector cells (10^6 in 0.5 ml) were mixed with ^{51}Cr-labeled target cells (10^5 in 0.2 ml) and then the heat-inactivated antiserum to these target cells (0.2 ml of various dilutions) was added. The mixtures were placed in conical plastic tubes, 10 $\times$ 64 mm (20), and supplemented with 0.1 ml medium to a total volume of 1 ml. Controls without added antiserum were included in all experiments. All assays were carried out in duplicate. The reaction mixtures were incubated for 20 hr. After incubation, 1 ml cold phosphate-buffered saline was added, tubes were centrifuged at 365 $\times$ g for 1 min, and 1 ml supernatant was carefully removed. Then the radioactivity of the supernatant was counted in a well type γ counter (21). The results were expressed as percentage of specific lysis as calculated by the following formula

$$\% \text{ Specific lysis} = \frac{\text{Experimental } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}}{\text{Maximal } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}} \times 100$$

Maximal ^{51}Cr release was determined by acetic acid lysis of target cells. Spontaneous ^{51}Cr release was 4% or less in all experiments. Cytotoxicity results are expressed as means of duplicates, within which variations rarely exceeded $\pm$2.5%.

ANTIBODY-DEPENDENT C′-MEDIATED LYSIS

Fresh, noninactivated immune maternal antiserum at various dilutions was added (0.2 ml) to appropriate ^{51}Cr-labeled A, B, or RhD RBC (0.2 ml containing 10^5 cells) in conical plastic tubes, and the mixtures were made up to 1 ml with medium. After 20 hr of incubation C′-mediated lysis was assessed in the manner described above.

RESULTS

SPECIFICITY OF ADCMC TO RhD AND A AND B ANTIGENS

In order to investigate specificity, ADCMC experiments were performed using the commercial preparations of human hyperimmune anti-A, anti-B, and anti-D sera. When 10^5 O RhD RBC as target cells were mixed with appropriate concentrations of heat-inactivated anti-D serum and with 10^6 MPBL as effector cells, lysis was readily demonstrable after 20 hr of incubation (Fig. 1*A*). This lysis was specific for RhD cells, since Rh– RBC were not lysed under comparable conditions. The absence of lysis of Rh– cells was not due to a technical failure, since A Rh– RBC were lysed in the presence of anti-A serum (Fig. 1*B*).

When heat-inactivated anti-A and anti-B sera were compared for their ability to induce ADCMC of human RBC of different blood group types, lysis was observed only when the labeled RBC carried the appropriate antigens (Fig. 1*B*).

These experiments, all of which were repeated several times using the same antiserum, demonstrated that the specificity of ADCMC was determined by the specificity of the antiserum.

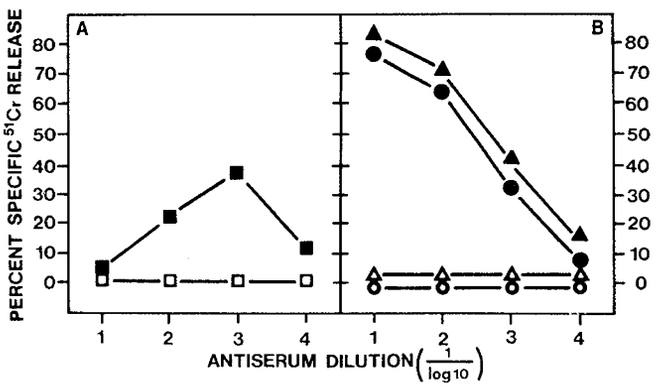

Fig. 1. Antibody-dependent, cell-mediated cytotoxicity induced by antisera against blood group antigens. Specific ^{51}Cr release estimated under standard experimental conditions, using various dilutions of the antisera. *A:* ■, anti-D serum with O RhD target cells; □, anti-D serum with A Rh− or B Rh− target cells. *B:* ▲, anti-A serum with A Rh− target cells; △, anti-A serum with B Rh− target cells; ●, anti-B serum with B Rh− target cells; ○, anti-B serum with A Rh− target cells. Mononuclear peripheral blood leukocytes alone or any of the antisera alone did not induce significant specific lysis. The results shown are those of a single representative experiment.

Table 1. *Antibody-dependent, cell-mediated cytotoxicity (ADCMC) of human erythrocytes using various antisera within rhesus system*

Antiserum used, 1/1,000 dilution	% ADCMC on target RBC with following phenotypes			
	ccddee	CcDee	CCDee	ccDEE
Anti-c	41	4	0	7
Anti-C	0	0	2	0
Anti-D	0	17	15	28
Anti-e	0	0	0	0
Anti-E	0	0	0	0

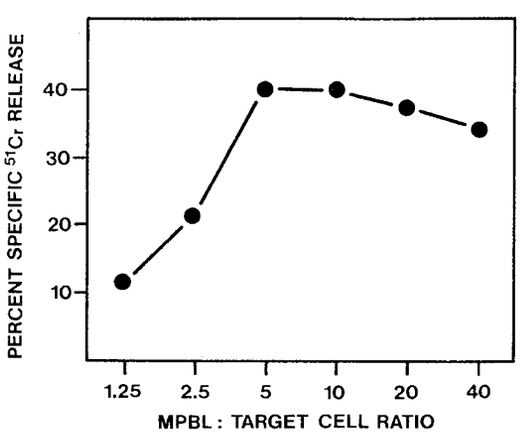

Fig. 2. Dependence of Antibody-dependent, cell-mediated cytotoxicity on the number of effector cells. Increasing numbers of mononuclear peripheral blood leukocytes (*MPBL*) were incubated with 10^5 RhD target cells, sensitized with anti-D serum (1/1,000).

ADCMC INDUCED BY ANTIBODIES TO OTHER Rh ANTIGENS

Various labeled RBC were incubated with MPBL and a 1/1,000 dilution of commercial anti-c, anti-C, anti-D, anti-e, and anti-E sera (Table 1). Definite lysis of homozygous Rh−cc RBC was achieved using the anti-c serum, whereas heterozygous RhDCc RBC were lysed very weakly. Anti-C, anti-e, and anti-E sera induced no or very little lysis. Control RhD RBC were lysed with anti-D serum. No evidence for nonspecific lysis was obtained in these experiments, which were performed three times with similar results.

CONDITIONS FOR ADCMC USING ANTI-D SERUM

Lysis was related to MPBL number in the range of 0.125–0.5 × 10^6 cells (MPBL:RBC ratio 1.25–5), but reached a plateau in the range of 0.5–4 × 10^6 cells (MPBL:RBC ratio 5–40) (Fig. 2). Similar levels of lysis (15–39%) were observed in nine other experiments.

Almost 90% of the final specific lysis was achieved in the first 6 hr of incubation (Fig. 3).

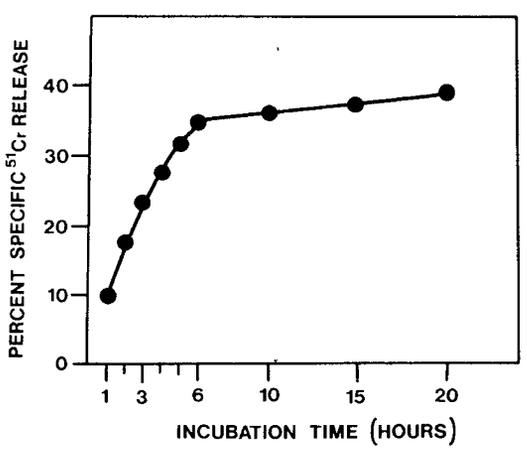

Fig. 3. The effect of time of incubation on antibody-dependent, cell-mediated cytotoxicity. Cultures were of 10^6 mononuclear peripheral blood leukocytes added to 10^5 RhD erythrocytes, sensitized with anti-D serum (1/1,000).

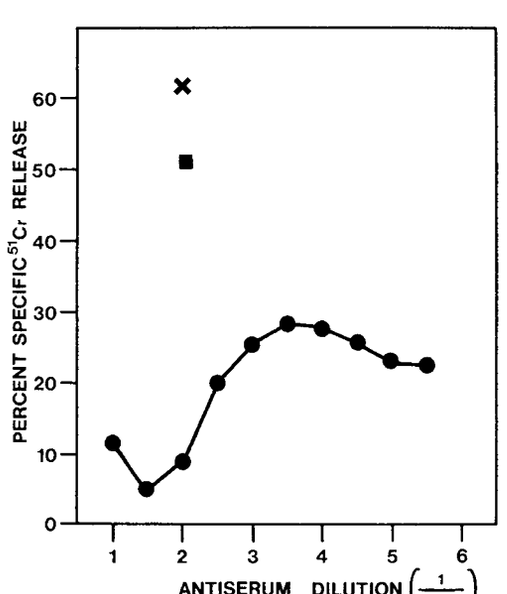

Fig. 4. Inhibition of antibody-dependent, cell-mediated cytotoxicity by high concentrations of anti-D serum. ●, standard experimental conditions. Target cells: 10^5 O RhD RBC. Effector cells: 10^6 mononuclear peripheral blood leukocytes. Commercial anti-D serum at the indicated dilution; ×, pretreatment procedure: target cells incubated for 30 min with a 1/1,000 dilution of antiserum and then washed twice before the 20 hr of incubation; ■, specific lysis obtained with anti-D serum (1/1,000 dilution), ultracentrifuged at 10 000 × *g* for 4 hours.

ADCMC was inhibited in the presence of high concentrations of anti-D serum (see examples in Fig. 1*A* and Fig. 4). This inhibition may be due to immunoglobulin aggregates in the antiserum. When target cells were incubated for 30 min with anti-D serum and washed twice before the addition of the effecor cells, more lysis was observed (Fig. 4). Increased lysis was also observed when an aliquot of the anti-D serum was ultracentrifuged before use (Fig. 4).

Table 2. *Assay of ability of Sephadex G-200 immunoglobulin fractions of commercial anti-D serum to induce antibody-dependent, cell-mediated cytotoxicity (ADCMC) of human RhD erythrocytes*[1]

Fraction	% ADCMC at dilution				
	1/10	1/100	1/1,000	1/10,000	1/100,000
Whole serum	0	13	27	20	5
IgM	0	0	0	n.d.[1]	n.d.
IgG	2	12	9	3	1

[1] n.d.: not determined.

Table 3. *Comparison of activity of maternal isoimmune sera in indirect Coombs' test and induction of antibody-dependent, cell-mediated cytotoxicity (ADCMC)*

Case	Type of isoimmunization	Maternal serum	
		Indirect Coombs' titer	% ADCMC[1]
1	RhD	1/256	28.4 (1/1,000)
2	RhD	1/256	31.2 (1/1,000)
3	RhD	1/16,384	26.1 (1/1,000)
4	RhD	1/16,384	25.9 (1/1,000)
5	RhD	1/16,384	33.8 (1/1,000)
6	RhD	1/16,384	40.9 (1/1,000)
7	RhD	1/32,768	38.1 (1/1,000)
8	RhD	1/65,536	38.6 (1/1,000)
9	RhD	1/262,144	41.0 (1/1,000)
10	RhD	1/524,288	42.2 (1/1,000)
11	OA	1/1,024	61.1 (1/100)
12	OA	1/1,024	68.1 (1/10)
13	OA	1/2,048	68.3 (1/10)
14	OB	1/2,048	58.4 (1/100)
15	OB	1/1,024	71.1 (1/10)

[1] Heat-inactivated maternal immune sera on adult red blood cells under standard experimental conditions. The antiserum dilution at which maximal lysis occurred is indicated in parentheses.

Commerical anti-D serum was separated on a Sephadex G-200 column into IgG and IgM fractions. No lysis was seen with the IgM fraction under standard experimental conditions. The IgG fraction did induce lysis, but less strongly than whole serum (Table 2).

ADCMC AND C'-MEDIATED LYSIS BY MATERNAL IMMUNE SERA

ADCMC was induced by 10 sera from mothers with RhD isoimmunization and in all instances was similar to that induced by commercial anti-D sera (Table 3). Maternal sera from three cases of OA and from two cases of OB isoimmunizations also induced ADCMC (Table 3). No correlation was found between the titer in the indirect Coombs' test and the percentage of lysis in ADCMC.

Direct (C'-mediated) lysis was observed when A or B group RBC were incubated for 20 hr with maternal noninactivated immune anti-A or anti-B serum at dilutions of 1/10 or 1/100. On the other hand, no hemolysis of RhD RBC occurred with maternal noninactivated immune anti-D serum. In all controls using heat-inactivated maternal anti-A, anti-B, and anti-D sera no direct lysis occurred. Usually, ADCMC was detectable using heat-inactivated serum at one or two doubling dilutions beyond the C'-mediated hemolytic concentrations for noninactivated serum (Table 4).

ADCMC OF NEWBORN RBC SENSITIZED *IN VIVO*

Cord RBC were collected from infants with fetal-maternal blood group incompatibilities. After washing, the cells were labeled and used as target cells. It was found that cells from RhD incompatibility were often lysed by MPBL from normal donors without addition of any further immune serum (Table 5, Direct ADCMC). From Table 5 it can also be seen that there was some correlation between the degree of lysis observed by direct ADCMC and the titer obtained in the direct Coombs' test. The two cases with the lowest Coombs' titers did not show lysis by the direct ADCMC technique. As a group the children who required two exchange transfusions had a higher percentage of lysis than the others.

No direct ADCMC was observed in the five cases of ABO incompatibilities. Their direct Coombs' titers were between 1/16 and 1/64.

Nevertheless, in all instances the heat-inactivated maternal sera induced ADCMC of the corresponding newborn target RBC, the amount of lysis (data not shown) being similar to that on adult RBC (Table 3).

AUTOLOGOUS MONOCYTES FROM NEWBORNS AS EFFECTOR CELLS IN ADCMC

RhD-sensitized cord blood RBC from *case 4*, with direct Coombs' titer of 1/4096, were lysed by autologous MPBL used at three concentrations. Comparable lysis was obtained with unrelated donor's MPBL (Fig. 5). Three other ADCMC tests, in which autologous and isologous effect cells were compared, are shown in Table 5.

Table 4. *Comparison of complement (C')-mediated and cell-mediated lysis induced by maternal isoimmune sera from three cases of fetal-maternal incompatibility*

Fetal-maternal incompatibility	% Specific antibody-dependent C'-mediated lysis[1]				% Specific ADCMC[2]			
	Antiserum dilution				Antiserum dilution			
	1/10	1/100	1/1,000	1/10,000	1/10	1/100	1/1,000	1/10,000
OA	72.1	68.4	0	0	68.3	65.5	38.1	24.7
OB	69.2	61.3	0	0	71.1	63.9	41.4	26.4
RhD	0	0	0	0	7.7	17.1	33.4	14.8

[1] Noninactivated maternal immune antiserum.

[2] ADCMC: antibody-dependent, cell-mediated cytotoxicity. Heat-inactivated maternal immune antiserum.

Table 5. *Susceptibility of newborn red blood cells to direct antibody-dependent, cell-mediated cytotoxicity (ADCMC)*

Case	Type of isoimmunization	No. of exchange transfusions	Direct Coombs' titer	% Direct ADCMC
1	RhD	0	1/16	0
2	RhD	1	1/64	0
3	RhD	1	1/4,096	22.2
4	RhD	1	1/4,096	25.0[1]
5	RhD	1	1/4,096	26.0[1]
6	RhD	2	1/8,192	39.6
7	RhD	2	1/8,192	38.7
8	RhD	1	1/16,384	42.2[1]
9	RhD	2	1/131,072	37.5
10	RhD	2	1/524,288	37.1[1]
11	OA	1	1/32	0
12	OA	0	1/32	0
13	OA	1	1/64	0
14	OB	0	1/16	0
15	OB	0	1/32	0

[1] Data given for autologous effector cells only.

Table 6. *Dependence of autologous antibody-dependent, cell-mediated cytotoxicity (ADCMC) on presence of phagocytic mononuclear peripheral blood leukocytes (MPBL)*

Treatment of MPBL	% Specific ^{51}Cr release					
	40:1[1]	20:1	10:1	5:1	2.5:1	1.25:1
None[2]	32.2	39.4	33.4	27.5	9.1	5.0
Carbonyl iron[3]	1.3	4.8	4.4	2.8	2.7	2.7

[1] MPBL to target cell ratio.
[2] Differential white cell count; 15–20% monocyte content.
[3] Differential white cell count; 2–3% monocyte content.

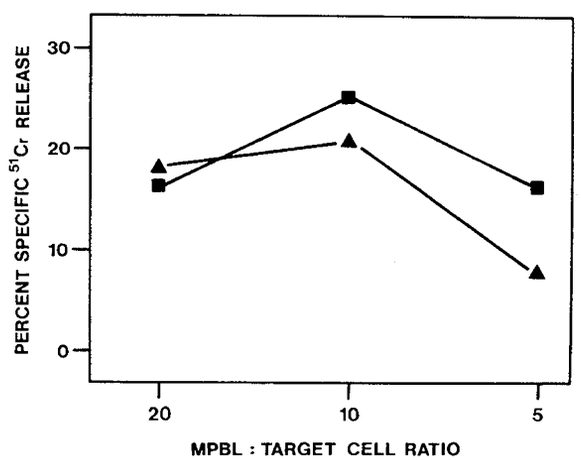

Fig. 5. Specific antibody-dependent, cell-mediated cytotoxicity induced by autologous and isologous effector cells on O RhD RBC sensitized *in vivo*. Target cells were 10^5 *in vivo* sensitized cord blood O RhD RBC. Mononuclear peripheral blood leukocytes were added at the ratios shown. ▲, unrelated donor O RhD MPBL; ■, autologous MPBL.

Monocytes were removed from a newborn MPBL suspension (*case 8* of RhD fetal-maternal incompatibility) by the carbonyl iron technique (13). ADCMC activity against cord blood RBC (sensitized *in vivo*) was strongly reduced by this procedure, suggesting that monocytes were the main effector cells in this ADCMC system (Table 6).

DISCUSSION

Our results show that nonimmune MPBL specifically lyse ^{51}Cr-labeled human erythrocytes sensitized *in vitro* or *in vivo* with antibodies directed against blood group antigens. In addition to ABO and RhD specificities, an antiserum against the rhesus phenotype cc also induced lysis of RBC of this phenotype. However, antisera to Cc, CC, ee, and EE induced little or no lysis of target cells bearing these antigens.

Isologous and autologous monocytes were found to act as effector cells for both *in vitro* and *in vivo* sensitized RBC. After separation of a commercial anti-D serum on a Sephadex column, only the IgG fraction was found to have appreciable ADCMC activity, in agreement with other studies (8). In all cases specificity of lysis corresponded to the specificity of the isoantiserum employed.

Most release of ^{51}Cr occurred during the first few hours of incubation with MPBL. Assessment of lysis at various MPBL to target cell ratios further suggested that the effector cells were quite efficient, since appreciable ^{51}Cr release was detected at ratios of about 1:1. In the absence of granulocytes, monocytes are the effector cells in this system (Table 6) (8, 9, 13). It is possible that actual effector to target cell ratios are of the order of 0.1–0.2:1. Our studies do not, however, exclude the possibility that granulocytes may also kill sensitized human erythrocytes (13, 15).

We also studied ADCMC in cases of fetal-maternal incompatibility. The mechanism of hemolysis in this situation is still unclear. In OA and OB incompatibility, antibodies are found which can induce C′-mediated lysis. The antibodies of Rh incompatibility, however, do not induce this lysis mechanism *in vitro*. Thus cell-mediated mechanisms may be more important in Rh hemolytic disease (1–4, 11).

The following observations are compatible with the possibility that ADCMC could operate *in vivo*. (*1*) Monocyte-mediated ADCMC of presensitized RBC can be obtained with immune sera against the specificities A, B, RhD, and cc which are involved in hemolytic disease of the newborn. The antibodies active in this system are probably of IgG class. (*2*) The sera of sensitized mothers and cord blood contain antibodies active in the ADCMC test. (*3*) There was some overall correlation between the severity of the hemolytic disease and the percentage of lysis induced in the direct ADCMC assay. Further work is needed, performed at various effector cell to targe cell ratios, to provide better information in this regard and to compare the prognostic values of either the direct Coombs' test or the direct ADCMC assay. (*4*) Effector cells are present in the newborn circulation.

SUMMARY

Antibody-dependent monocyte-mediated cytotoxicity can destroy fetal erythrocytes. All of the components necessary for this destruction are present in newborn infants with fetal-maternal incompatibility.

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Adenosine triphosphate
erythroblasts
fetus
hemoglobin synthesis
hypoxia
liver

Effect of Hypoxia on Erythroblasts from Avian Fetal Liver: Adenosine Triphosphate Levels and Hemoglobin Synthesis

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Extract

A system for the isolation and functional evaluation of fetal liver erythroblasts is described. Isolated erythroblasts were prepared from 14-day embryonic avian livers and incubated at various oxygen tensions (0, 5, 12, and 95%). The concentration of ATP in erythroblasts remained constant for at least 4 hr at 37°, but was rapidly reduced by incubation in nitrogen. Protein synthesis as measured by L-[^{14}C]leucine incorporation into cell protein occurred at a linear rate in 5%, 12%, and 95% oxygen, whereas little protein synthesis occurred at 0% oxygen. The effect of hypoxia on the type of hemoglobin synthesized was studied in this system by isolating the hemoglobin A, hemoglobin D, and hemoglobin H fractions and determining the incorporation of L-[^{14}C]leucine. The major fraction, hemoglobin A, contained most of the radioactivity; smaller amounts were present in hemoglobin D and hemoglobin H, respectively. The relative proportion of each hemoglobin synthesized was not altered by oxygen from 5% to 95%. These results argue against a direct effect of oxygen on the type of hemoglobin synthesized at this stage of development.

Speculation

Early in fetal development nonerythropoietin mechanisms for the regulation of erythropoiesis may exist. The hypoxic stimulus to erythropoiesis is mediated through erythropoietin in definitive erythroblasts, but may have a direct effect in primitive erythroblasts.

A major step towards therapy for sickle cell anemia and the thalassemia syndromes, the most common of the hereditary hemolytic anemias, would be an understanding of erythropoiesis during development. In man the pattern is probably like that in the avian embryo, where erythropoiesis develops from a primitive to a definitive cell with associated changes from embryonic to adult hemoglobins (11, 12, 14, 22, 36, 43). A similar developmen-