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The Pathogenesis and Prevention of Rh Immunization

ALVIN ZIPURSKY, M.D., F.R.C.P.[C]* and
LYONEL G. ISRAELS, M.D., F.R.C.P.[C],† *Winnipeg, Man.*

RH immunization of Rh-negative women during or subsequent to pregnancy is a response to the presence of fetal red cells in the mother. It has been the goal of several groups, including our own, to define this process of isoimmunization in Rh-negative women and to develop a rational program for its prevention.

Our earlier studies[1] and those of others[2, 3] showed that fetal erythrocytes frequently are found in the blood of pregnant women during pregnancy; immediately following labour, larger numbers of fetal erythrocytes are found in the mother's blood than during the pregnancy.[4] Therefore it has been suggested that the delivery period is the time of maximum risk of immunization.[4] This proposal is supported by the recent studies of Woodrow *et al.*,[5] who showed that large transplacental hemorrhages at the time of birth are frequently followed by the development of Rh immunization. Furthermore, analysis of their data indicates that there is a direct relationship between the volume of circulating fetal cells in the mother immediately after delivery and the probability of subsequent Rh immunization. However, more than 50% of the women who subsequently became immunized had few or no fetal cells demonstrable in their blood at delivery. Cohen and Zuelzer[6] in their study of 127 Rh-negative women found that only one of the eight who subsequently developed antibodies had had a large transplacental hemorrhage at delivery. They suggested that Rh-negative women are at risk throughout pregnancy and that the delivery process is relatively unimportant in the pathogenesis of Rh isoimmunization. Similarly we have shown that primary isoimmunization may follow a pregnancy in which fetal erythrocytes had not been demonstrated post partum.[7]

The possibility that Rh immunization can result from the relatively small quantities of fetal erythrocytes entering the maternal circulation before labour is supported by the observation that Rh-negative subjects produced Rh antibodies following repeated injections of 0.1 ml. of Rh-positive fetal erythrocytes.[7] This provides support for the concept that isoimmunization can result either from the small volumes (less than 0.1 ml.) of fetal cells which frequently enter the maternal circulation throughout pregnancy[1, 2] or from a large transplacental hemorrhage at delivery. In the present study the relative importance of these two possible mechanisms in the pathogenesis of Rh isoimmunization is examined. A program of prophylaxis in both antepartum and postpartum women has been undertaken. On the basis of these observations, we report here our initial studies in both antepartum and postpartum women on the development of a program for the prevention of Rh immunization.

Methods

In our early studies fetal cells were demonstrated by a modification of the acid elution technique of Kleihauer, Braun and Betke.[8] More recently we have employed the original technique because, as pointed out by Cohen *et al.*,[2] the distinction between fetal and adult hemo-

From the Departments of Pediatrics and Medicine, University of Manitoba, Winnipeg, Manitoba.
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*Professor and Head, Department of Pediatrics, McMaster University, Hamilton, Ontario.
†Professor of Medicine, University of Manitoba, Winnipeg.
Reprint requests to: Dr. A. Zipursky, Department of Pediatrics, Faculty of Medicine, McMaster University, Hamilton, Ontario.

TABLE I.—INCIDENCE OF FETAL CELLS IN THE BLOOD OF Rh-NEGATIVE WOMEN IMMEDIATELY AFTER DELIVERY

Scan count*	Estimated volume of fetal red blood cells in maternal circulation (ml.)†	Total group		Baby Rh+		Baby Rh—	
		Number	%	Number	%	Number	%
0	0 - 0.02	980	82.4	613	82.3	267	82.5
1 - 4	0.02 - 0.1	149	12.5	97	13.0	52	11.7
5 - 20	0.1 - 0.2	34	2.8	20	2.7	14	3.1
>20	>0.2	27	2.3	15	2.0	12	2.7
Total		1190	100.0	745	100.0	445	100.0

*Number of cells found during a 10-minute scan of two blood smears.
†An approximation based on the number of cells seen per 10-minute scan (see Methods).

globin-containing cells is much clearer. In our hands, the *in vitro* sensitivity of the two techniques is similar. Recently we have further modified the technique to distinguish more clearly between fetal erythrocytes and leukocytes (see Appendix).

Using *in vitro* mixtures of fetal and adult cells, the lower limit of detection is one fetal cell in 100,000 adult red cells.[1] This is equivalent to about 0.04 ml. of fetal blood or 0.02 ml. of fetal erythrocytes in the maternal circulation. This order of *in vitro* sensitivity is valid *in vivo* as we have been able to detect fetal cells in four adults injected with 0.01 ml. of fetal cells. This is a 10-fold increase in sensitivity over that reported by us previously.[1]

We have used the counts of *in vitro* mixtures to calibrate the assay for estimating the volume of fetal cells in the maternal circulation.[1] We estimate that 5 to 20 cells per 10-minute scan represents 0.1-0.2 ml. of fetal cells and that more than 20 cells represents greater than 0.2 ml. in the maternal circulation. Woodrow *et al.*[5] consider that a "fetal-cell score" of five by their method represents 0.25 ml. of fetal blood or approximately 0.1 ml. of cells. It would appear that the techniques have the same sensitivity.

The presence of antibodies and their titres were determined by techniques referred to previously.[1] Erythrocyte-survival studies were done by either the ^{51}Cr technique or by the persistence of fetal cells by the acid elution method. With the latter technique the survival of fetal erythrocytes in the circulation of adult volunteers has been found to range from 56 to 105 days.[9]

RESULTS AND DISCUSSION

A. *The Pathogenesis of Rh Isoimmunization. The Incidence of Fetal Erythrocytes in the Circulation of Postpartum Women and the Subsequent Development of Rh Antibodies*

Blood for fetal cells was drawn within the first 24 hours post partum from 1190 Rh-negative women. The number of such cells seen during a 10-minute scan is designated in the tables as the "Scan Count". The subjects were then arbitrarily divided into four groups in accordance with the scan counts. Although it is only semi-quantitative, the method readily permits the identification of a group of women who have received unusually large numbers of fetal cells. In the present study we have arbitrarily selected a scan count of five or more cells (representing approximately 0.1 ml. or more of fetal erythrocytes in the maternal circulation) as indicative of a "large" transplacental hemorrhage. Table I shows that of the total group of 1190 Rh-negative women, 5.1% had a scan count of five or more. The incidence of scan counts in excess of five or more was 4.7% in those women with Rh-positive babies and 5.8% in those with Rh-negative babies. These differences are not statistically significant. The influence of ABO incompatibility on the incidence of fetal cells in the maternal circulation is shown in Table II. High scan counts (5 cells or more) clearly occurred more often in the ABO compatible group (59 out of 948) than in the incompatible group (2 out of 242). The incidence of lower scan counts (1 to 4 cells) was also greater in the ABO compatible group ($\chi^2 = 17.2$; $p < .001$).

TABLE II.—THE EFFECT OF ABO INCOMPATIBILITY ON THE PRESENCE OF FETAL CELLS POST PARTUM

Scan count	Volume of fetal red blood cells in maternal circulation (ml.)	ABO compatible		ABO incompatible	
		No. of women	%	No. of women	%
0	0 - 0.02	756	79.7	224	92.6
1 - 4	0.02 - 0.1	133	14.0	16	6.6
5 - 20	0.1 - 0.2	33	3.5	1	0.4
>20	>0.2	26	2.8	1	0.4
Total		948	100.0	242	100

These studies indicate that fetal erythrocytes in quantities of 0.1 ml. or more are found in 5.1% of postpartum women, and in highest frequency in ABO compatible pregnancies. This incidence is compared to those reported by others in Table III. The incidence of fetal cells in an unselected group in quantities greater than 0.10 ml. in the present series was compared to the postpartum cases reported by Woodrow

TABLE III.—INCIDENCE OF "LARGE" NUMBERS OF FETAL CELLS IN THE BLOOD OF WOMEN IMMEDIATELY AFTER DELIVERY

Author	Number of women	Approximate volume in ml.*	Number of instances	%	
A. *ABO blood group of baby and mother not considered*					
Woodrow *et al*[5] (Table II)	200	>0.12	17	8.5	$\chi^2 = 3.68$ (not significant)
Present study	1190	>0.10	61	5.1	
Woodrow and Finn[4]	692	>0.12	92	13.3	$\chi^2 = 37.4$ p = <.001
B. *Baby and mother ABO compatible*					
Woodrow et al.[5] (Table III)	216	>0.12	26	12.0	$\chi^2 = 8.78$ p = <0.01
Present study	948	>0.10	59	6.3	
Cohen *et al.*[2] (Table VI)	622	>0.20	52	8.4	$\chi^2 = 25.1$ p = <0.001
Present study	948	>0.20	26	2.8	

*In each instance the approximate volume was deduced from the fetal cell count. Woodrow *et al.*[5] and Cohen *et al.*[2] record their so-deduced volumes as volumes of whole blood. These have been converted to volumes of cells by dividing their figures by two.

et al.[5] (Table III); the two groups were not statistically different ($\chi^2 = 3.68$; $p > .05$). A more recent publication by Woodrow and Finn[4] reports that 19.8% of 692 postpartum samples had fetal-cell scans greater than three and 13.3% had fetal-cell scans of five or more (which they interpret to represent 0.25 ml. of blood[5]). This incidence of 13.3% is significantly greater ($\chi^2 = 37.4$; $p < .001$) than that of the present series. When ABO compatible pregnancies are compared (with reference to quantities greater than 0.1 ml.), the present series differs significantly from the early series of Woodrow *et al.*[5] ($\chi^2 = 8.78$; $p < .01$). In comparing hemorrhages of 0.2 ml. or more, Cohen *et al.*[2] reported a much higher incidence than the present series ($\chi^2 = 25.1$; $p < .001$). Because of the unstandardized and variable crude methods of estimating volumes of fetal cells in the maternal circulation, such comparisons are difficult. Nevertheless it is possible that the incidence of "large" transplacental hemorrhages may vary in different centres and these observations may, in part, explain the relatively high proportion of isoimmunization due to "large" transplacental hemorrhages in the Liverpool study as compared with our series *(vide infra).*

The time at which transplacental hemorrhage occurs has been studied recently by Woodrow and Finn.[4] They concluded that large transplacental hemorrhages (fetal-cell score of five or more) are more likely to occur at delivery than at any other time during pregnancy. Our own findings in a group of 87 women followed up during the last month of pregnancy (Table XIII) do not support their contention. In this series, labour played an insignificant role in the production of fetal hemorrhage. A similar conclusion was reached by Cohen *et al.*,[2] who found that of 11 women whose blood contained an estimated 0.2 to 5.0 ml. of fetal cells after delivery, seven had had similar amounts present from 1 to 43 days earlier. However, they did find that fetal erythrocytes enter the maternal circulation in greater frequency as pregnancy progresses. They found that the incidence of fetal cells in the first, second and third trimesters increased progressively from 6.7 to 15.9 to 28.9%. Similar observations have been made by Clayton *et al.*[10] and by Woodrow and Finn.[4] It would appear therefore that transplacental hemorrhage is more likely to occur in the last weeks of pregnancy. The relative importance of labour in the frequency and quantity of transplacental hemorrhage is not yet clear.

Woodrow *et al.*[5] consider that those women who have received "large" (greater than 0.25 ml.) quantities of fetal blood at or near the time of labour are at the greatest risk of developing antibodies and that the incidence of immunization increases in direct relationship to the volume of fetal blood in circulation post partum. It is not known, however, what proportion of Rh immunization results from such "high risk" pregnancies. Thus Cohen and Zuelzer[6] reported that of eight women who had developed antibodies after a pregnancy, only one had had a "large" quantity of fetal erythrocytes in her circulation post partum. We previously reported two women in whom antibodies had developed in the absence of a "large" transplacental hemorrhage.[7]

To determine the relative importance of "large" and "small" bleeds in the pathogenesis of Rh immunization, 573 women (whose babies were Rh positive) have been observed post partum for the development of anti-D antibodies. Blood was drawn from these women 4

TABLE IV.—RELATIONSHIP OF THE VOLUME OF FETAL ERYTHROCYTES IN THE MATERNAL CIRCULATION POST PARTUM AND THE SUBSEQUENT DEVELOPMENT OF Rh ANTIBODIES

Cell scan	*Approximate volume of fetal cells*	*Women immunized/total group*	*% immunized*
0 - 4.....	0 - 0.1	16/538	3.0
>4.....	>0.1	5/35	14.3

to 12 months post partum, and the serum was tested for antibodies. The results shown in Table IV demonstrate two significant points. The first is that those women whose fetal-cell scan immediately post partum was greater than four, had an incidence of Rh immunization of 14.3% compared to an incidence of 3% in the group with lower cell scans. This supports the contention of Woodrow *et al.*[5] regarding the relationship between the size of the fetal bleed and chance of subsequent Rh immunization and therefore the existence of a "high risk" group.

TABLE V.—WOMEN WHO DEVELOPED ANTIBODIES POST PARTUM

	Scan count		*Parity* P	*Parity* M
Infant ABO-compatible..	0 - 4	13	4	9
	>4	5	3	2
Infant ABO-incompatible....	0 - 4	3	1	2
	>4	0		

However, the data in Table IV also show that most Rh immunizations followed a pregnancy in which the postpartum cell scan was four or less, since 16 of the 21 immunized women were in this category. Of the women who were immunized, eight were primiparae, and five of these did not have a "large" volume of fetal erythrocytes in their blood post partum (Table V). In these women, the primary immunizing event must have been a transplacental hemorrhage which was either very small or occurred well before delivery.

These data, along with those of Cohen and Zuelzer[6] and of Woodrow *et al.*[5] as reported by McConnell,[11] are summarized in Table VI. Thus of 51 women who developed antibodies following delivery, fetal cells were not demonstrable post partum in 22. An additional 13 had "small" quantities of cells and would therefore not have been recognized as a "high risk" group. Sixteen of the 39 women did have "large" bleeds demonstrable post partum. Thus, in 69% of the women in the three series, the development of antibodies must have resulted from an antigenic stimulus prior to labour or perhaps as a result of a "small", frequently undetectable hemorrhage during labour. Woodrow *et al.*[5] have criticized the data of Cohen and Zuelzer because the latter authors did not state whether their subjects were primiparae or multiparae, since if they had been multiparae the appearance of antibodies could have reflected a secondary stimulus. Eight patients in our study were primiparae (Table V) and in five there was no evidence of a "large" transplacental hemorrhage post partum. Furthermore, all of the subjects of Woodrow *et al.*[5] (Table V) were primiparae and 7 of 13 had not had a "large" bleed post partum. Therefore, it would appear that Rh isoimmunization occurs in women either as a result of a "large" transplacental hemorrhage at delivery or probably more often as a result of smaller, sometimes undetectable bleeds which occur at any time in pregnancy. Woodrow *et al.*[5] based their original studies of immunological protection on the identification of the "high risk" postpartum group. In the light of our experience this would protect only a minority of the Rh-negative women at risk. Any program of prophylaxis of Rh immunization must be based on these considerations and should include not only the "high risk" group but all Rh-negative women at risk.

B. *Studies on the Prevention of Rh Immunization. The Use of Rh-Immune Globulin*

RESULTS

Rh isoimmunization in volunteers receiving Rh-positive cells can be prevented by the administration of anti-Rh containing gamma globulin or serum.[12, 13] This suggested that a similar approach could be used for the prevention of

TABLE VI.—RELATIONSHIP BETWEEN THE PRESENCE OF FETAL ERYTHROCYTES IN THE CIRCULATION POST PARTUM AND THE SUBSEQUENT DEVELOPMENT OF ANTI-Rh ANTIBODIES
Quantity of fetal erythrocytes found in the maternal circulation post partum

	"Large" *No. of women*	*Approx. vol.*	*"Small" (1-4)* *No. of women*	*Approx. vol.*	*None* *No. of women*
Cohen and Zuelzer*[6]	1	(0.2 ml.)	3	(0.02-0.2 ml.)	4
McConnell[11]	10	(>0.12 ml.)	7	(<0.12 ml.)	5
Present series	5	(>0.1 ml.)	3	(<0.1 ml.)	13

*The original data in the authors' articles were expressed as numbers of fetal cells from which the amount of fetal *blood* was estimated. These estimates have been converted in this table to ml. of fetal *erythrocytes* by dividing by two.

Rh isoimmunization in pregnant women. Preliminary results of such studies have been reported by others and suggest that passive immunization, post partum, with anti-Rh antibody can prevent Rh isoimmunization.[11, 14] We have undertaken similar studies. Specifically we have: (1) examined the biological activity of a preparation of anti-Rh globulin ("Rh-Immune Globulin"); (2) studied the effect of this agent on newly delivered Rh-negative women whose blood contained Rh-positive fetal cells; and (3) studied the use of this preparation in Rh-negative women during pregnancy.

The results of these investigations follow.

1. The Biological Activity of a Preparation of Anti-Rh (D) Gamma Globulin ("Rh-Immune Globulin")

"Rh-Immune Globulin" was prepared by the Connaught Medical Research Laboratories, University of Toronto, from plasma obtained by repeated plasmaphereses of three Rh-negative women. We selected as our plasma donors women who had had at least one fetal death due to Rh hemolytic disease. We thought the response of the fetus a better index of the quality of antibody than the test-tube titre. Their antibody titres before and after plasmaphereses are shown in Table VII. Three lots were used successively in the present study. The first (Lot 1-1) contained 5% and the second and third (Lots 2-1 and 3-1) 16% gamma globulin. Antibody titres of Lot 3-1 are shown in Table VIII. The titres obtained by several laboratories are also listed in Table VIII. They demonstrate the difficulty in comparing antibody titres from one laboratory to those of another. The antibody titre of Lot 2-1 was similar to that of 3-1 but Lot 1-1 was lower, with a titre in albumin of 1 out of 320 and indirect Coombs of 1 out of 640. The anti-D content of Lot 3-1 was measured for us by Dr. N. C. Hughes-Jones (St. Mary's Hospital, London, England) and found to be 190 μg. per ml. The gamma globulin was administered as a deep intramuscular injection. No side effects were associated with its use.

TABLE VII.—Antibody Titres in the Plasma Used as a Source of Anti-Rh (D) Gamma Globulin

	Before plasmaphereses				*After plasmaphereses*		
		*Antibody titre**				*Antibody titre**	
Donor	*Date*	*S*	*A*	*Number of plasmaphereses*	*Date*	*S*	*A*
A	June, 63	1	128	17	Aug./64	1	64
B	June, 63	1	256	5	Aug./64	1	64
Br	Dec. 63	1	64	9	Aug./64	1	256

*S and A represent the reciprocal of the saline and albumin titres respectively.

TABLE VIII.—Anti-D Titrations of Lot 3-1, Rh Immune Globulin

	Technique	
	Albumin	*Indirect Coombs*
Rh Laboratory (Winnipeg)	1/1024	1/6400
Medical Research Council Laboratories (London)	1/5000	1/20,000
Dade Laboratories (Florida)	1/4000	
Commonwealth Serum Laboratories (Australia)	1/16,000	1/4000
St. Mary's Hospital (London)		1/4000

a. *Anti-D Levels in Recipients Following an Injection of Rh-Immune Globulin*

The appearance of anti-Rh (D) antibody in the circulation following intramuscular injection of 20 ml. of Lot 1-1 is shown in Fig. 1. Anti-D was found first after four hours, reached maximum levels in two days and persisted for over six weeks. The maximum levels of anti-D activity achieved after various doses are shown in Table IX.

TABLE IX.—Maximum Anti-D Levels in Circulation Following Intramuscular Injection of Rh-Immune Globulin

Rh immune globulin (ml.)		*Maximum anti-D level attained**			
Lot 1-1 (5%)	*Lot 2-1 (16%)*	*Saline*	*Papain*	*Albumin*	*Indirect Coombs*
20.0		0	8	2	4
5.0		0	4	2	2
1.0		0	Tr	0	Tr.
	1.5	0	+	0	±
	0.4	0	0	0	0

*Antibody level is expressed as the reciprocal of antibody titre.
Tr = trace.
+ = antibody present, demonstrable in whole serum only.
± = antibody possibly present.

b. *Effect of Rh-Immune Globulin on the Life Span of Rh-Positive Fetal Red Cells*

(i) *In Women After Delivery*

The survival of fetal cells, as determined by the acid-elution technique, was followed in 11

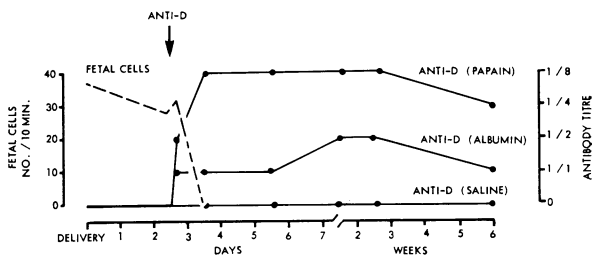

Fig. 1.—Patient S. Fetal erythrocytes were found in the circulation post partum and on repeat examination. Twenty ml. of 5% anti-D gamma globulin were given at 86 hours post partum. The disappearance of fetal cells as well as the appearance of antibodies is shown.

TABLE X.—SURVIVAL OF Rh-POSITIVE, ABO-COMPATIBLE, FETAL ERYTHROCYTES IN POSTPARTUM WOMEN AFTER INJECTION OF Rh-IMMUNE GLOBULIN

Subject	Time of injection (hours post partum)	Volume of injection (ml.)		Scan count					
				Prior to injection	After injection (hours)				
		Lot 1-1	Lot 2-1		24	48	72	96	120
S.	86	20		30*	0	0			
P.	64	20		2.6%†	82	1	0		
F.	120	5		530	63	0			
V.	24		1.5	31	2	6	0		
Ra.	51		1.5	46	0				
R.	27		1.5	52		5	0		
B.	40		1.5	215	140	0			
Mc.				190	253	65	1		
A.	140		1.5	784	420	28	0		
Pl.	72		1.5	3.2%†	2.0%	2.3%	2.0%	2.0%†	
D.	40		0.4	22			15	0	
St.	48		0.4	140			110		0

*All results are recorded as scan counts except for the pre-injection sample in patient "P" and all samples in patient "Pl", where results are recorded as percentage of cells in the patient's blood that were fetal.

†A second dose of 1.5 ml. (Lot 2-1) was given and when next tested six days later, no cells were found.

women who had received Rh-Immune Globulin post partum. The survival of fetal cells in one of these (Mrs. S.) is shown in Fig. 1 and all results are shown in Table X. When quantities of 5.0 ml. or more of Lot 1-1 or 1.5 ml. of Lot 2-1 were given, fetal cells were, with two exceptions, gone by 48 hours. In Mrs. R. fetal cells were still present at 48 hours but had disappeared by 72 hours. In Mrs. Pl. 3.2% of the cells in her circulation were fetal when she received her first injection of Rh-Immune Globulin after delivery. As this did not remove the Rh-positive cells over the next four days, she was given a second injection. Fetal cells had disappeared from her circulation when she was next studied six days later. The smallest dose administered, 0.4 ml. of Lot 2-1, appeared to remove fetal cells at a slower rate in two subjects since cells were still demonstrable at 72 hours, although they had disappeared when the blood was examined at 96 and 120 hours.

(ii) *In Males Receiving Untagged Cells*

Eighteen Rh-negative men were each given 2 ml. of Rh-positive cord erythrocytes intravenously. Forty-eight hours later, six received 5.0 ml. and six 1.0 ml. of Lot 1-1 Rh-Immune Globulin and six control subjects received 1.0 ml. of commercial gamma globulin. Fetal cell counts were done at 24-hour intervals beginning 48 hours before injection of the gamma globulin. In the group receiving 5 ml. there was a rapid disappearance of fetal cells, most of them being gone by 48 hours. The disappearance was slower in the group receiving 1.0 ml., although it was more rapid than in the control. At one week no fetal cells were demonstrable in those receiving either 1.0 or 5.0 ml. anti-D gamma globulin, whereas in five of the six controls such cells were seen in numbers from 9 to 73% of the original (mean survival was 43%). Two weeks after injection there were no fetal cells demonstrable in the control group.

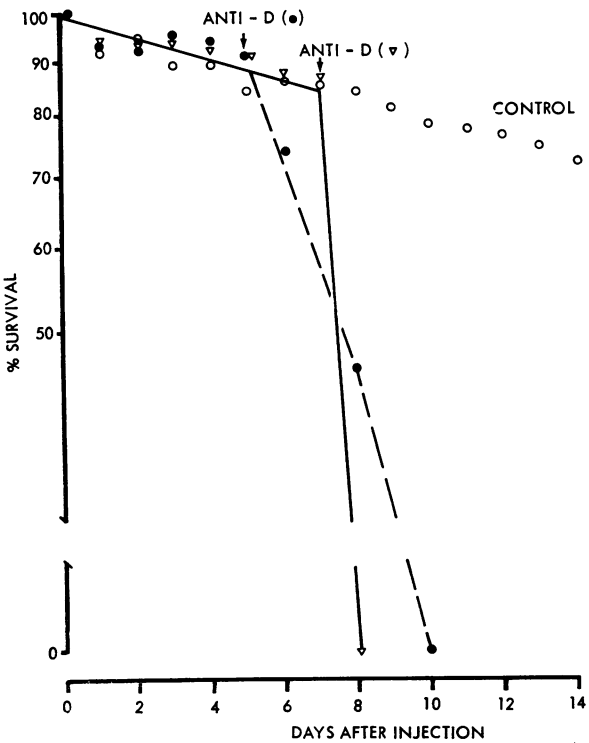

Fig. 2.—The survival of ^{51}Cr-labelled Rh-positive cord erythrocytes in the circulation of three Rh-negative subjects. Each subject received 1.6 ml. cord erythrocytes on day 0. 0—control; ●—received 0.5 ml. 5% Immune Globulin on day 5; Λ—received 2.0 ml. 5% Rh Immune Globulin on day 8. A fourth subject (not shown), who received 1 ml. on day 5, had an erythrocyte survival that did not differ significantly from that of the control.

The reason for the disappearance of the fetal cells in this group is not clear, although it may be related to immunization of some of these subjects.

(iii) *Males Receiving ^{51}Cr Labelled Cells*

Four Rh-negative men each received, from one donor, 1.6 ml. of the same Group O Rh-positive compatible cord erythrocytes tagged with 100 microcuries of ^{51}Cr. All subjects were studied for five days to determine the cell survival over that period. On day 5, one subject received 1.0 ml. and one received 0.5 ml. of Lot 1-1 Rh-Immune Globulin. A third subject was given 2.0 ml. on day 8 while the fourth was retained as a control. The results (Fig. 2) show that the injected cells were rapidly removed from the circulation by an injection of 2.0 ml. of gamma globulin and at a slower rate by 0.5 ml. In one case not shown in this figure, a dose of 1.0 ml. was ineffective (the ^{51}Cr survival was identical with the control), suggesting that removal may not be solely a function of dose. Further studies using smaller volumes of fetal cells were done and the results are shown in Table XI.

TABLE XI.—RH-IMMUNE GLOBULIN (CONNAUGHT) AND CLEARANCE OF RH-POSITIVE CORD ERYTHROCYTES FROM THE CIRCULATION OF RH-NEGATIVE SUBJECTS

Experiment	*Volume of cells (ml.)*	*Volume of 16% gamma globulin (ml.)*	*^{51}Cr survival (half-life) days*
He	0.5	0.5	<1
T	0.5	0.5	<1
He	0.34	0.1	2
SH	0.34	0.02	3.5

In one subject, He., repeated ^{51}Cr erythrocyte survival studies were performed following the injection of 0.5 ml. of Rh-Immune Globulin (Fig. 3). As indicated in Table XI, erythrocyte survival immediately after the injection of 0.5 ml. gamma globulin was less than 24 hours. The survival of 0.5-ml. volumes of cord erythrocytes was still less than three days two months later, less than six days at three months, and it was only after four months that the ^{51}Cr survival of Rh-positive cord erythrocytes approached normal (Fig. 3). Assuming a half-life of Rh-immune globulin in circulation of 30 days, it can be estimated that 98 days after the injection of 0.5 ml. of Rh-Immune Globulin the amount remaining in circulation would be equivalent to an injection of approximately 0.06 ml. of Rh-Immune Globulin.

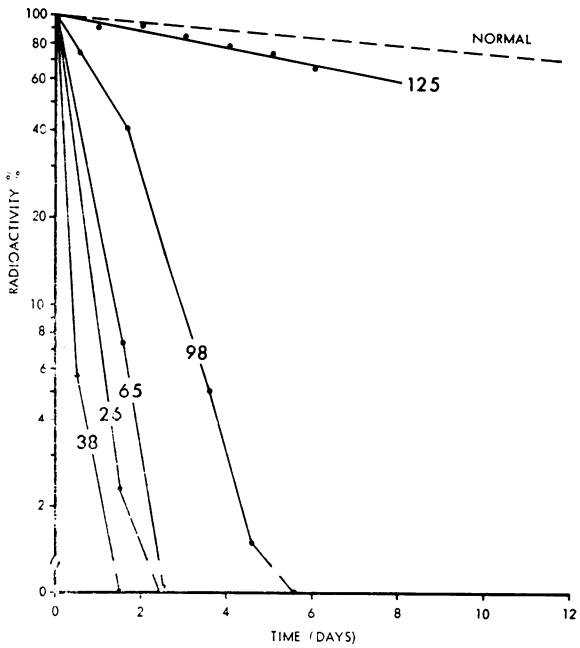

Fig. 3.—^{51}Cr survival studies at intervals following the administration of 0.5 ml. Rh-Immune Globulin. The dashed line represents a hypothetical survival curve for normal blood. The solid lines represent survival curves of ^{51}Cr-labelled erythrocytes (0.5 ml.) given at 26, 38, 65, 98 and 125 days following the administration of Rh-Immune Globulin.

Thus these studies (Table XI and Fig. 3) indicate that 0.02 ml. to 0.06 ml. (3.8 to 11.4 micrograms of anti-D antibody) of Rh-Immune Globulin can effectively shorten the life span of 0.3 to 0.5 ml. volumes of Rh-positive cord erythrocytes. This is comparable to the data of Mollison and Hughes-Jones,[15] who found that one microgram of anti-D antibody decreased the half-life ^{51}Cr survival of 0.3 ml. of cells to 100 hours. In two experiments where five micrograms were given, the half-life was 39.5 and 43.5 hours respectively.

c. *The Effect of Rh-Immune Globulin in Preventing Primary Rh Isoimmunization in Rh-Negative Subjects*

This study is the continuation of that described in section b (ii). Eighteen Rh-negative men who did not have Rh antibodies were each given 2.0 ml. of Rh-positive cord blood intravenously. Subjects 1 to 8 received blood from one placenta, subjects 9 to 13 from a second and subjects 14 to 18 from a third. Forty-eight hours later, six (1 to 5 and subject 17) were injected intramuscularly with 5 ml. and six (6 to 10 and subject 16) with 1.0 ml. of Lot 1-1 Rh-Immune Globulin (5%), while six controls received 1.0 ml. of commercial gamma globulin (16%).

Three of the controls developed anti-D (Table XII), whereas none of the protected did. Six

months after the initial injection 13 of the subjects received a second injection of 2.0 ml. of Rh-positive cells. Repeat injections were not given to four of the six in the 5.0 ml. group or to one of the six in the 1.0-ml. group. None of those who did not already have anti-D developed it during the three months following this second stimulus, but a significant rise in antibody titre took place in the three immunized subjects who already had anti-D (Table XII).

TABLE XII.—ANTIBODY RESPONSE OF THREE MALE SUBJECTS

Date	Day		Fetal red blood cells (vol.)	Anti-D antibody											
				Subject H.				Subject M.				Subject Mc.			
				S.	A.	P.	I.C.	S.	A.	P.	I.C.	S.	A.	P.	I.C.
Nov. 3, 1964	0		2.0 ml.	—	—	—	—	—	—	—	—	—	—	—	—
Dec. 10	38			—	—	—	—	+	—	—	—	—	—	—	—
Dec. 24	52			1	—	1	—	+	+	2	+	—	—	—	—
Jan. 25, 1965	84			1	1	2	1	1	1	4	2	—	—	—	—
Feb. 25	111			2	2	2	2	—	2	4	2	—	—	—	—
March 25	139			1	2	2	2	—	1	1	1	—	—	—	—
April 22	168	*Day*		1	4	8	4	—	—	2	1	—	—	Tr.	—
May 20	196	0	2.0 ml.	1	4	8	8	—	—	1	—	—	—	Tr.	Tr.
May 27		7		16	16	32	32	8	64	256	128	4	1	8	2
June 3		14		16	32	128	64	1	64	256	128	1	16	64	64
June 17		28		16	64	256	128	1	64	256	128	2	16	32	32
July 17		58		2	128	256	128	1	32	256	128	1	8	32	32
Aug. 22		92		1	128	512	128	1	64	256	128	1	4	8	8

*Antibody levels are expressed as the reciprocal of the antibody titre.
S = Saline, A = Albumin, P = Papain, I.C. = Indirect Coombs.
—No antibody demonstrable.
Tr. Trace of antibody.

The antibody responses (Table XII) of the three immunized men deserve individual comment. The response of subject H. is a "standard" response; thus, no antibody was demonstrable on day 38, but one, demonstrable by the saline and papain techniques, was present by day 52, and one demonstrable by all four techniques was present by the day 84. Thereafter the titre rose slowly to a peak at days 168 to 196. The secondary response was brisk, the saline titre falling away after day 28. In subject M, the primary response began a little earlier, but followed the same pattern of development, although at a lower level; by day 139 the response was beginning to fade and by day 196 the antibody could be demonstrated only by the papain method. If on this day this man had been tested only by the indirect Coombs technique, he would have been considered as unimmunized. Interestingly, in spite of the generally weak primary response, the secondary response was even brisker than that of subject H. In subject Mc. the only evidence of primary immunization was a very weak papain and Coombs reaction on day 196, and yet his response to the second stimulus is a classical, though low-level, secondary response. Had only the albumin technique been used in subjects M. and Mc., primary immunization would not have been detected on day 196.

These observations emphasize an important consideration in any study dealing with Rh immunization and its prevention. Reliance cannot be placed on a single method of detection when antibodies are being sought; further, serial examinations for antibodies should be carried out. The final proof that primary immunization has not occurred is failure of the recipient to give a secondary response reaction to a second dose of antigen.

2. The Use of Rh-Immune Globulin to Prevent Primary Rh Immunization in Women Whose Blood Contains Rh-Positive Fetal Erythrocytes After Delivery

Fifteen newly delivered Rh-negative women whose blood had scan counts of five or more, and whose babies were ABO-compatible and Rh-positive, received Rh-Immune Globulin and were followed up for three or more months post partum (nine have been followed for six or more months post partum). They received 1.5 ml. of 16% Rh-Immune Globulin with the exception of two who received 0.4 ml. of 16% and two received 5 and 20 ml., respectively, of 5% Rh-Immune Globulin. Antibodies are not demonstrable in any of these 15 women. To date 35 women who had scan counts of five or more after delivery and did not receive Rh-Immune

Globulin have been tested at four or more months post partum. Five had antibodies (Table IV).

3. The Use of Rh-Immune Globulin During the Third Trimester of Pregnancy

The data outlined in the previous sections suggest that many if not most cases of primary Rh immunization result from transplacental passage of fetal cells at some time before the onset of labour. Accordingly, it seemed necessary to develop a technique whereby Rh-Immune Globulin could be given during pregnancy. That this seemed feasible was suggested by the findings noted earlier, which indicated that small doses of Rh-Immune Globulin were adequate to destroy fetal erythrocytes and prevent Rh immunization (see Section B.1). Nevertheless, it seemed possible that even these small quantities of a lethal antibody might cause harm to the fetus. Accordingly, before proceeding with the injection of pregnant women we attempted to simulate the condition in the fetus, by examining the effect of Rh-Immune Globulin on the life span of Rh-positive erythrocytes in Rh-positive subjects.

Two Rh-positive infants, R. (aged 2 months and weighing 8 pounds) and M (aged 18 months and weighing 18 pounds), were each given 5.0 ml. of ^{51}Cr-tagged autologous blood. Forty-eight hours later each received 0.5 ml. of Rh-Immune Globulin (Lot 1-1) while M. received a second dose of 0.5 ml. one week later. The erythrocyte ^{51}Cr life span in M. is shown in Fig. 4. The calculated half-life for red-cell survival in R. and M. was found to be 21 and 31 days, respectively. (A half-life of 21 days is normal for the erythrocytes of an infant of 2 months.[17])

Thus, quantities of antibody far in excess of those which a fetus would likely receive if the mother were given a "protective" dose of Rh-Immune Globulin did not appear to shorten erythrocyte life span. We concluded that it would be safe to give such doses of antibody to mothers during pregnancy. Still proceeding with caution, we at first gave the globulin at various times during the last trimester (Series A).

We started by giving a single dose of 0.4 ml. one week before delivery. Later we increased this to a single dose of 1 ml. one week before delivery. Our final program consisted of 1 ml. given at what was judged to be the 28th week followed by two 0.4 ml. doses four weeks apart.

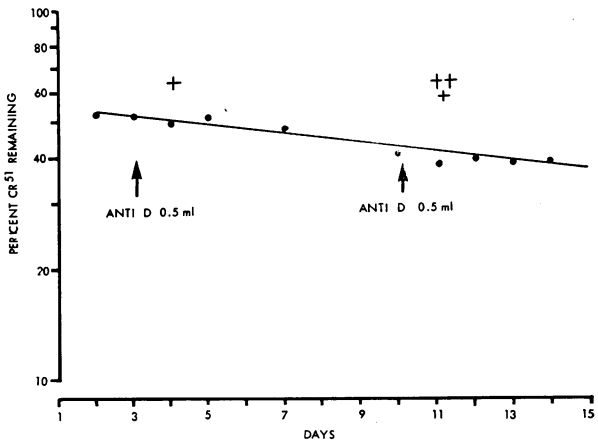

Fig. 4.—^{51}Cr survival of autologous cells in an 18-month, Rh-positive experimental subject. On day 3, the subject received 0.5 ml. of 5% anti-D (Rh-Immune Globulin). This was repeated on day 10.

+=Direct Coombs test weakly positive.
++=Direct Coombs test strongly positive.

a. Series A

(i) *Anti-D Levels Following the Injection of Anti-D Gamma Globulin During Pregnancy*

Forty-seven pregnant women received Rh-Immune Globulin and were studied serially thereafter for antibodies in their serum. Only two of the women given a dose of 0.4 ml. had demonstrable antibodies in their serum, but antibodies were demonstrable in three of seven, of those receiving a single injection of 1.0 ml. In those women receiving multiple injections, antibodies were demonstrable in all but two of the 23 cases studied. In all cases in which they were found, antibodies were demonstrable only by the papain technique and were never more than "weakly positive".

(ii) *The Effect on the Fetus of Anti-Rh Administration to the Mother*

Immediately after birth the following hematological studies were performed on all babies: hemoglobin concentration, hematocrit, reticulocyte count, white blood count and differential. All babies were followed for evidence of hyperbilirubinemia in the first five days of life. Thirty-five of the babies were Rh-positive. The direct Coombs test was "weakly positive" in five of these infants. Two of these five were ABO-incompatible, which may explain the positive test. In one of these (born one day after receiving 0.4 ml. anti-Rh gamma globulin) the bilirubin rose to 14.0 mg. % by day 3 (this was considered to represent a case of ABO-incompatibility—hemolytic disease). All infants were well and there was no evidence of hematological ab-

normalities related to the administration of Rh-Immune Globulin to the mother.

(iii) *The Incidence of Fetal Erythrocytes in the Circulation of Mothers Receiving Rh-Immune Globulin*

The blood of 34 women who subsequently delivered Rh-positive infants was examined for fetal cells immediately before the administration of Rh-Immune Globulin. In six or 17.5%, fetal cells were demonstrable. In five of these, studied within a week after injection, fetal cells were no longer found.

Fetal cells were found for the first time following injection in four of 10 women receiving 0.4 ml., in one of seven receiving 1.0 ml. and in five of the 18 who received multiple injections.

(iv) *The Development of Antibodies in Women Receiving Rh-Immune Globulin*

Twenty-five women have been followed up for longer than four months (20 for more than six months). None have developed antibodies. Six of the group received 0.4 ml., one 0.8 ml. and the remainder 1 ml. or more. Four of the group were ABO-incompatible.

b. SERIES B

In the second antepartum study (Series B), Rh-negative pregnant women were divided into three groups. One received 1.5 ml. Rh-Immune Globulin at the 36th week of gestation; one received 1.5 ml. immediately postpartum and one acted as the control group.

(i) *The Incidence of Fetal Erythrocytes*

These observations during the last month of pregnancy are shown in Table XIII. It is evident that large volumes of fetal erythrocytes are less frequently found in the circulation of women who received 1.5 ml. Rh-Immune Globulin at the 36th week of gestation. The frequency of small transplacental bleeds did not differ significantly in the groups studied.

(ii) *The Newborn Infant*

All infants were hematologically normal and with one exception the Coombs test was negative; in that patient, the mother was group O and the infant was group A.

(iii) *The Development of Antibodies*

Blood samples obtained six months postpartum, or longer, were examined for anti-D antibodies. In the unprotected group 1 of 21 developed an anti-D antibody. In the group who received Rh-Immune Globulin at delivery (20) and at 36 weeks' gestation (20) none developed antibodies. ABO-incompatibility between mother and fetus was found in two of 21 unprotected, four of 20 injected post partum and five of 20 injected at 36 weeks.

TABLE XIII.—SCAN COUNTS OF FETAL CELLS IN CIRCULATION DURING THE LAST MONTH OF PREGNANCY IN SERIES B

a. The entire group at 36 weeks and the untreated thereafter

		Scan counts			
	Total	*0*	*5*	*5 - 20*	*20*
36 weeks	87	75	8	4	0
37 weeks	54	45	7	1	1
38 weeks	43	33	5	4	1
Labour: 1st stage	47	38	7	1	1
After 3rd stage	57	44	8	3	1

b. Women who received 1.5 ml. Rh-Immune Globulin at 36 weeks

		Scan counts			
	Total	*0*	*5*	*5 - 20*	*20*
37 weeks	24	20	4	0	0
38 weeks	16	12	4	0	0
Labour: 1st stage	15	15	0	0	0
After 3rd stage	24	19	5	0	0

GENERAL DISCUSSION

The present studies were undertaken on the thesis that Rh isoimmunization during pregnancy results from the transplacental passage of fetal erythrocytes and that active immunization can be prevented by passive immunization with anti-Rh antibodies. This hypothesis has been presented by others[12, 13] and preliminary observations by these groups offer support for the rationale of this approach.[11, 14]

The Rh-Immune Globulin used in the present study has been prepared in three lots; initially a 5% preparation (Lot 1-1) and later 16% gamma globulin preparations (Lots 2-1 and 3-1). All three have incomplete antibody activity demonstrable *in vitro* (Table VIII) and *in vivo* (Table IX, Fig. 1). The absence of complete (saline) antibodies is in part due to the selection of donors (Table VII) who had very low levels of saline antibodies and in part to the method of preparation of concentrate which removes most of the macroglobulin (IgM) antibodies. The antibody titre of our anti-Rh gamma globulin, as we have measured it, is considerably lower than that used by others.[5, 13] The donors had been selected not primarily because of their antibody titre but because in all cases the antibody appeared to be capable of producing severe hemolytic disease. This was evidenced by a history of fetal death (due to Rh disease) in all women.

We have demonstrated in volunteers that as little as 0.02 ml. of Rh-Immune Globulin can significantly shorten the life span of 0.34 ml. of Rh-positive cord erythrocytes. These findings are supported by the observations in pregnant women whose blood contained fetal erythrocytes (Table X). In these cases it appeared that 1.5 ml. was effective in removing small quantities of fetal cells; it is likely also that in two women, D. and St., a dose of 0.4 ml. caused a less distinct, but significant shortening of erythrocyte life span. However, when the quantity of Rh-positive erythrocytes in circulation is large, greater volumes of antibody must be used to effect removal. This is clearly evident in subject Pl. (Table X). This woman was found to have 3.2% fetal cells in her blood, which would represent about 125 ml. of fetal blood in her circulation. This quantity of blood was not removed by the administration of 1.5 ml. anti-Rh gamma globulin (Lot 2-1), but did disappear after a second dose. In subject P. a large dose of 20.0 ml. (Lot 1-1) quickly removed a similar volume of fetal erythrocytes. These observations suggest that the removal of small quantities (2.0 ml. or less) of fetal Rh-positive erythrocytes from the maternal circulation, in most instances, was effected by the administration of 0.4-1.5 ml. (Lot 2-1) anti-Rh gamma globulin.

Although it is evident that this preparation destroys fetal Rh-positive erythrocytes, its biological effectiveness must be judged by its ability to prevent primary isoimmunization of Rh-negative subjects by Rh-positive erythrocytes. That these two effects (on life span and on antigenicity) are interrelated has been suggested by the original studies of Clarke *et al.*[12] Nevertheless, it remained to be determined whether our anti-Rh gamma globulin preparation could prevent primary Rh isoimmunization in experimental subjects and pregnant women. The 12 "protected" subjects in Section B (1,c,) did not develop primary immunization. Seven were restimulated six months later and did not show a secondary or booster response. Three of the six "unprotected" subjects developed antibodies and showed a classical secondary response when restimulated. Thus doses of 1.0 ml. and 5.0 ml. of 5% gamma globulin were effective in preventing primary immunization. This quantity of antibody is considerably less than that used by Woodrow *et al.*[5] and Freda, Gorman and Pollack[13] to prevent primary isoimmunization. However, neither of these groups attempted to establish the minimum effective dose. Recently, however, Clarke *et al.*[16] presented evidence which suggested that as little as 0.5 ml. anti-D gamma globulin is capable of preventing Rh immunization. Gorman's data suggest that 100 μg. of anti-D could prevent Rh immunization following the injection of 6.0 ml. of Rh-positive erythrocytes.[17] His findings are comparable to those of the present study, in which it was found that as little as 1.0 ml. of 5% Immune Globulin prevented Rh immunization. This volume is assumed to contain approximately 60 μg. anti-D on the assumption that its content would be 5/16 of that in the 16% preparation. Gorman,[17] however, found that 10 μg. was not protective. This suggests that the minimum dose for the prevention of Rh immunization is between 10 and 100 μg. of anti-D antibody.

Our studies to date in postpartum women suggest that our preparation is effective in preventing primary immunization in Rh-negative mothers. Thus 15 mothers who had had "large" transplacental hemorrhages and received anti-Rh gamma globulin have been followed up for more than three months and none have developed antibodies. In contrast, 35 women in the present series who had "large" transplacental hemorrhages have been followed up for more than four months and five have already developed antibodies.

The Liverpool group have held that most cases of Rh immunization result from a "large" transplacental hemorrhage occurring during labour. They have identified these women and studied the effectiveness of their preparation of anti-D containing gamma globulin in preventing immunization. Their most recent results in this high-risk group are encouraging and indicate that these women can be protected.[18] However, the evidence presented in this paper (Section A) and by others[6] suggests that identification and protection of the "high risk" group will not prevent the majority of cases of Rh immunization. We believe that Rh immunization can result from the smaller bleeds which occur so frequently during pregnancy. For this reason we considered the possibility of treating women during pregnancy. It is evident from our studies that such a procedure is feasible and does not harm the fetus and that "protective" doses of Rh Immune Globulin can be given safely during pregnancy.

Freda, Gorman and Pollack[14] have approached the problem in another way. They reasoned that since antibodies rarely appear during the immunizing pregnancy, passive immunization should be effective if given immediately post partum. In their study, anti-Rh antibodies are administered to Rh-negative women post partum if the infant is Rh-positive, ABO-compatible, irrespective of the presence or absence of fetal cells in the maternal circulation. Their most

recent findings strongly support this approach. Thus 37 of 318 controls became immunized, whereas none of 369 women who had received protection developed antibodies.[17]

These results all represent postpartum follow-up studies in which women have been observed for the appearance of Rh antibodies. The lack of antibody formation strongly suggests that Rh immunization has been prevented; however, for the reasons given, these studies must be continued into subsequent pregnancies to determine that the protected women have not indeed been sensitized and whether or not antibodies will appear during their next Rh-positive pregnancy. However, preliminary observations from both the Liverpool and Columbia groups[14, 17] indicate that the protected women do *not* develop antibodies during a subsequent pregnancy. It would appear therefore that the protection is real and that postpartum passive immunization with Rh antibodies is an effective means of preventing Rh immunization. Accordingly many centres are now actively studying the value of their own anti-Rh antibody preparation in the prevention of Rh immunization.

In Canada, the Connaught Medical Research Laboratories of the University of Toronto have prepared Rh-Immune Globulin, as used in the present study. This material is now being used in a number of centres throughout Canada as part of a co-ordinated program which will lead to the availability of a simple and practical means of preventing Rh immunization.

Summary The relation between transplacental hemorrhage and the development of Rh immunization has been studied.

Rh immunization developed more frequently in women whose blood contained relatively large volumes of fetal erythrocytes post partum. Nevertheless this high-risk group accounted for only a small proportion of women developing Rh antibodies post partum. It is suggested, therefore, that all Rh-negative women, whose infants are Rh-positive, ABO-compatible, are at risk of developing antibodies.

Rh-Immune Globulin (Connaught), with anti-D activity demonstrable *in vitro* and *in vivo*, can destroy Rh-positive erythrocytes and appears to prevent Rh immunization in experimental subjects and postpartum women.

Rh-Immune Globulin has been given to Rh-negative women, ante partum and post partum, with no ill effect. It is likely, therefore, that this preparation will prove to be an effective means of preventing Rh immunization.

Résumé Cet article aborde la question de la relation entre l'hémorragie transplacentaire et l'apparition de l'iso-immunisation Rh.

L'iso-immunisation Rh apparaît plus fréquemment chez les femmes dont le sang contient des quantités relativement importantes d'hématies fétales durant le post-partum. Pourtant, ce groupe de sujets, bien qu'il constitue un "risque élevé", n'a représenté qu'une faible proportion des femmes qui ont présenté des anticorps Rh au cours du post-partum. On estime donc que toutes les femmes à Rh-négatif, dont les enfants ont un Rh-positif avec compatibilité ABO, courent le risque de former des anticorps.

La globuline immunisante Rh (Connaught), dotée d'une activité anti-D décelable *in vitro* et *in vivo*, peut détruire les érythrocytes Rh-positifs et permet de prévenir l'iso-immunisation Rh chez des sujets d'expérience et chez les femmes durant le post-partum.

On a administré la globuline immunisante Rh à des femmes Rh-négatives, tant *ante-partum* que *post-partum*, sans déclencher de réactions défavorables. Il est donc probable que ce produit se révélera comme un moyen efficace de prévenir l'iso-immunisation Rh.

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APPENDIX

THE ACID ELUTION TECHNIQUE FOR THE DEMONSTRATION OF FETAL ERYTHROCYTES

Reagents:

1. *Hematoxylin solution*

Hematoxylin crystals	5 g.
Alcohol—absolute	50 ml.
Ammonium or potassium alum	100 g.
Mercuric oxide	2.5 g.
Distilled water	1000 ml.

Dissolve the hematoxylin in the alcohol and the alum in the water with the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible. Remove from heat and add the mercuric oxide slowly. Reheat until it becomes dark

purple, remove from flame immediately and plunge the vessel into a basin of cold water until cool.

The stain is ready for use as soon as it cools. Addition of 2 to 4 ml. of glacial acetic acid per 100 ml. of solution increases the intensity of the nuclear stain. Filter before use.

2. *Eosin solution* (0.5%)

Eosin (water soluble, yellow shade) 5 g.
Distilled water..... 1000 ml.
Filter before storing.

3. *Buffer*

Prepare immediately before use:
75.4 ml. of 0.1M citric acid
24.6 ml. of 0.2M sodium phosphate
pH should be 3.2 - 3.3

Technique:

Fresh clotted blood is preferred; however, heparinized blood may be used. Alcohol-cleaned slides must be used. Mix, on the slide, two drops of serum with one drop of blood and prepare a thin blood smear. Air-dry for 30 - 60 minutes.

Slides are fixed in 80% alcohol for five minutes. Wash thoroughly under running cold tap water. Place in buffer (at 30°C.) for five minutes. Wash slides again under running tap water for five minutes. Stain for five minutes with eosin. Wash eosin off with running water. Stain slides with freshly filtered hematoxylin for two to three minutes. Wash and dry in air.

The Clinical Stages of Breast Cancer—What Do They Mean?

JAMES E. DEVITT, M.D., C.M., M.Sc., F.R.C.S.(Edin.), F.R.C.S.[C],
Ottawa, Ont.

DURING the last 15 years, every reported prospective trial[1-5] or retrospective review[6-11] of breast cancer patients treated at one centre has failed to show any advantage of radical therapy (either surgical or radiotherapeutic) over conservative treatment. These observations clearly contradict the traditional understanding of the behaviour of breast cancer. Consequently, it seemed desirable to study some of the features of carcinoma of the breast to see if more acceptable concepts could be developed. It was realized that such concepts would be general in nature, and qualified by the need to consider as one disease what may be different diseases presenting rather similar histological appearances.

The first parameter studied was the significance of regional lymph node metastases.[12] It was concluded that the poor prognosis associated with metastatic regional lymph nodes was not due to these metastases. Rather, both prognosis and metastases were evidence of the biological potential of the tumour. The possibility was considered that the regional lymph node metastases might not be important sources for the further spread of breast cancer.

Some observations concerning the clinical stage of the tumours when the patients were first seen—the "presenting clinical stage"—are now reported, and their significance is discussed.

From the Civic Hospital Division, Ottawa Clinic, Ontario Cancer Foundation, and the Division of Surgery, Ottawa Civic Hospital, Ottawa, Ontario.

Reprint requests to: Dr. James E. Devitt, Chairman, Department of Medical Education and Research, Ottawa Civic Hospital, Ottawa 3, Ontario.

MATERIAL AND METHOD

The material consists of all of the 1440 female patients reported to the Civic Hospital Division of the Ottawa Clinic of the Ontario Cancer Foundation, whose treatment for carcinoma of the breast was started between 1946 and 1961 inclusive. It seemed likely that these patients make up a typical population of the victims of breast cancer—for Canada at least.

Because some of the preoperative examination records were vague, the following retrospective method of staging was based on the pathological reports as to the measurement of the tumour size, and the presence of axillary lymph node metastases:

Stage I: The tumour was 5 cm. or less in size. There was no *peau d'orange* phenomenon, skin infiltration or ulceration (dimpling, skin "tethering" and nipple retraction were not considered as evidence of skin invasion). There was no fixation of the tumour to underlying tissues. No supraclavicular lymph nodes were palpable, and edema of the arm was absent. Histological examination of the excised axillary lymph nodes did not reveal metastases.

Stage II: The clinical signs accompanying the primary tumour were as in Stage I, but there were histologically proved axillary lymph node metastases. If these nodes were palpable clinically, they were not fixed to each other or to adjacent structures.

Stage III: The tumour was greater than 5 cm. in greatest diameter, and the patient had one or more of the clinical signs described as being absent in the first two stages.