Infective and anti-infective properties of breastmilk from HIV-1-infected women

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Human immunodeficiency virus type 1 (HIV-1) is transmitted mainly by cell-to-cell contact. We postulated that transmission of HIV-1 through breastmilk could be favoured by the presence of infected cells, by deficiency of anti-infective substances in breastmilk, or both factors.

215 HIV-1-infected women were enrolled at delivery in Kigali, Rwanda; milk samples were collected 15 days, 6 months, and 18 months post partum. HIV-1 IgG, secretory IgA, and IgM were assayed by western blot, for the latter two after removal of IgG with protein G. In the 15-day and 6-month samples, we sought viral genome in milk cells by a double polymerase chain reaction with three sets of primers (gag, pol, and env). HIV-1 infection in the offspring was defined according to serological and clinical criteria. At 15 days, 6 months, and 18 months post partum, HIV-1 specific IgG was detected in 95%, 98%, and 97% of breastmilk samples, IgA in 23%, 28%, and 41%, and IgM in 66%, 78%, and 41%. In children who survived longer than 18 months, the probability of infection was associated with lack of persistence of IgM and IgA in their mothers' milk (adjusted χ^2 for trend, p=0·01 for IgM and p=0·05 for IgA). The presence of HIV-1-infected cells in the milk 15 days post partum was strongly predictive of HIV-1 infection in the child, by both univariate (p<0·05) and multivariate analysis (p=0·01). The combination of HIV-1-infected cells in breastmilk and a defective IgM response was the strongest predictor of infection.

HIV-1 infection in breastfed children born to infected mothers is associated with the presence of integrated viral DNA in the mothers' milk cells. IgM and IgA anti-HIV-1 in breastmilk may protect against postnatal transmission of the virus.

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Introduction

Postnatal mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1) is well recognised.[1] In Rwanda, the estimated rate of HIV-1 transmission from mothers who acquired infection post partum to their infants ranged from 25% to 53%.[2] This high risk of HIV-1 transmission has been attributed to the high viral burden in primary infection.[3] In some cohorts of children born to HIV-1-infected women in developed countries, the probability of infection was higher for breastfed infants than for bottle-fed infants.[4,5] However, these observational studies were not designed to estimate the excess risk of transmission attributable to postnatal transmission from

women who had been infected before delivery, a problem that remains unsolved. The biological properties of milk from HIV-1-infected women have not been fully studied. Two reports have described the detection of HIV-1 antibodies of IgG, IgA, and IgM classes in breastmilk samples from infected women.[6,7] Both sample sizes were too small for any conclusion about possible effect of these antibodies on the transmission of HIV-1 to infants to be drawn.

Many animal retroviruses, like human T-cell leukaemia virus type 1, are mainly transmitted by cell-to-cell contacts.[8] We therefore postulated that postnatal transmission of HIV-1 through breastmilk, if it occurs, should be favoured by the presence in breastmilk of infected cells and deficiency of anti-infective substances such as specific antibodies. We report here our study of sequential breastmilk samples from 215 HIV-1-infected mothers in Kigali, Rwanda.

Subjects and methods

A prospective cohort study on perinatal transmission of HIV-1 started in Kigali, the capital of Rwanda, in November, 1988. All women giving birth at the maternity ward of the Centre Hospitalier de Kigali (CHK) were told by social workers of the objectives, constraints, and advantages of the study. Verbal consent was obtained from each woman who agreed to take part. 215 HIV-1 seropositive mothers and 217 seronegative mothers were enrolled with their newborn infants. Details of enrolment procedures are given elsewhere.[9] We concentrate in this paper on mother-infant pairs seropositive at the time of delivery.

After delivery, enrolled families were visited every 2 weeks by social workers, who collected information on the health of the children by means of standard questionnaires. The children and their mothers were examined by a physician every 3 months. When necessary, children were seen in the outpatient clinic or admitted to the CHK, and treated free of charge. At study entry none of the HIV-1-seropositive mothers met the World Health Organisation (WHO) clinical case-definition of acquired immunodeficiency syndrome (AIDS).[10] Except for histories of herpes zoster infection (reported in 9) and chronic cough, the frequency of signs and symptoms did not differ between HIV-1 seropositive and seronegative mothers.

Criteria used to classify children of seropositive mothers as infected with HIV-1, not infected, or with indeterminate status are drawn from a working group consensus on mother-to-child transmission of HIV.[11,12] The use of this classification is restricted to children who had reached or could have reached the age of 15 months by the time of analysis. It takes into account the HIV-1 antibody status at 15 months and, if the child died before that age, the presence or absence of signs and symptoms. A child was taken to be HIV infected if he or she had AIDS[13] at whatever age, died of an

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TABLE I—NUMBERS OF MILK AND BLOOD SAMPLES AVAILABLE
AT EACH TESTING POINT

—	Day 15	Month 6	Month 18
*Mothers followed in cohort**	210	195	151
Lactating women	208	184	119
Blood sample available for CD4/CD8 testing	188	ND	ND
Milk samples tested:			
By PCR	129	96	ND
For IgG anti-HIV-1	176	153	104
For IgA anti-HIV-1	162	142	95
For IgM anti-HIV-1	168	153	85

*Total enrolled minus those lost to follow-up, those who had died, and those whose babies had died.
ND = not done.

HIV-related cause, or was HIV-antibody positive at 15 months. A child was taken as not infected with HIV if he or she was HIV-antibody negative at 15 months or was lost to follow-up or died of an HIV-unrelated cause and was HIV-antibody negative at 9 months or beyond. All other children are classified as of indeterminate status.

Every 3 months, blood samples were taken from mothers and children. Serum samples were screened by an enzyme-linked immunoassay (Vironostika, Organon Teknika, Boxtel, Netherlands). All positive samples were confirmed by western blot (Du Pont, Wilmington, Delaware, USA). 15 days post partum, a 10 mL blood sample was obtained from the mothers. Mononuclear-cell subpopulations were quantified by means of an indirect immunofluorescence technique and monoclonal antibodies. A CD4/CD8 ratio below 0·5 was taken as the criterion for severe immunodeficiency.

We asked each lactating mother for 10–15 mL breastmilk 15 days, 6 months, and 18 months post partum. Milk samples were immediately centrifuged at 300 g. The lipid layer was removed with a pipette and lactoserum was stored at −20°C until testing. Cell pellets were washed twice in Coon's solution containing 1% bovine serum albumin, then stored at −70°C.

Milk HIV-1 IgG antibodies were detected by means of a standard western blot test (Novapath, Bio-Rad, Hercules, California, USA). Specific IgM antibodies were assayed by western blot with the avidin-horseradish peroxidase system (anti-IgM conjugate, μ-chain specific, diluted 1 in 500, Sigma Immunochemicals, Deisenhofen, Germany) and strips from Organon Teknika. Milk IgA antibodies were tested by western blot (Bio-Rad), by means of an anti-human-IgA, α-chain specific, alkaline-phosphatase conjugate diluted 1 in 500 (Sigma). This conjugate is most suitable for detection of secretory IgA. For each antibody test, the sample dilution used was 1 in 100. To improve the specificity and sensitivity of specific IgM and IgA detection, western blots were done after treatment of lactoserum samples with *Staphylococcus aureus* protein G (Pansorbin and Sansorbin, Calbiochem Corporation, La Jolla, California, USA) to remove IgG.[6,14] No residual IgG was detected by radial immunodiffusion (Norpartigen IgG, Behring, Marburg, Germany)[15] after this process. To ensure specificity, western blot assays were repeated on a subset of lactoserum samples positive for HIV-1 IgM, IgA, or both, after preincubation with serial dilutions of specific goat antibodies to human μ chain or α chain and demonstration of progressive inhibition of HIV-1 antibody detection by western blot.[14] For the detection of specific IgM and IgA antibodies, a positive sample was required to have reactive bands corresponding to HIV-1 p24 (major nucleocapsid protein), gp 120/160 (major envelope glycoprotein), or both, with or without additional bands.[6]

DNA from the mononuclear cells pelleted from the milk samples was extracted[2] and a double PCR was done with three pairs of HIV-1-specific oligonucleotide sequences in the *gag*, *env*, and *pol* regions of the HIV-1 genome.[2] As well as known negative controls, internal controls were included in each PCR run; these were 34 DNA samples extracted from cell pellets of milk samples collected in HIV-1-seronegative mothers from the comparative cohort (13 at 15 days and 21 at 6 months post partum). A PCR was judged
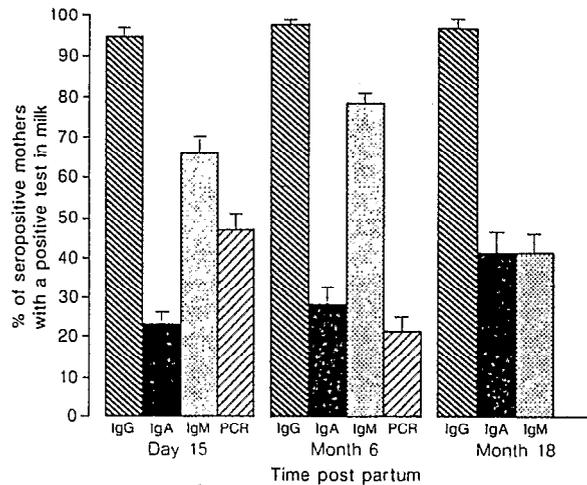
Detection of HIV-1 antibodies of IgG, IgA, and IgM classes and HIV-1-infected cells by PCR in milk samples.

Vertical bars = 95% CI

positive when at least two of the three primer pairs gave a detectable signal.

The Mantel-Haenszel χ^2 test, MacNemar test, adjusted and unadjusted χ^2 test for linear trend, two-tailed Fisher's exact test, and Student's *t* test were used for comparisons, with a significance level of 0·05. Unadjusted and Mantel-Haenszel weighted odds ratios were calculated to measure the strength of association when appropriate,[16] and their 95% CI were calculated by the Cornfield method. Exact confidence limits were also calculated when necessary. Finally, we constructed a multivariate logistic regression model in an attempt to explain the HIV-1 infection status of the children, which included maternal age, parity, history of adverse pregnancy outcome, maternal clinical status at delivery, method of delivery, sexually transmitted diseases diagnosed at the time of delivery, presence or absence of infected cells in milk by PCR 15 days post partum, persistence of HIV-1 IgM up to 18 months (3 positive samples *vs* less than 3), and the mother's CD4/CD8 ratio 15 days post partum.

Results

46 (21·1%) of the 218 children born to HIV-1-infected mothers were classified as HIV-1 infected, 140 (64·2%) as not infected, and 32 (14·7%) as indeterminate status.

15 days after delivery 72% of 188 HIV-1-seropositive mothers had CD4/CD8 ratios below 1·0, including 24% who had ratios below 0·5.[8] The proportion of mothers who gave milk samples was 85% on day 15, 83% at 6 months, and 87% at 18 months (table I). In some cases there was not enough lactoserum for us to do all three serological tests, but there was no difference in HIV-1 status between children whose mothers' milk was and was not tested. 47 (27%) of 176 samples collected at day 15 and 57 (37%) of 153 collected at month 6 had insufficient milk or insufficient

TABLE II—HIV-1 INFECTION STATUS OF CHILDREN WHO
SURVIVED LONGER THAN 18 MONTHS AND PERSISTENCE OF
MILK HIV-1 IGM

Milk samples with anti-HIV-1 IgM*	HIV-1-infected infants	HIV-1-uninfected infants	Mantel-Haenszel weighted odds ratio (95% CI)
0	5	6	1·00
1	6	11	0·90 (0·08–10·1)
2	7	21	0·28 (0·04–1·89)
3	3	25	0·16 (0·02–1·17)
Total	21	63	..

*Maximum 3, since milk was collected at day 15, month 6, and month 18.

TABLE III—RELATION OF HIV-1 INFECTION STATUS OF
CHILDREN TO PRESENCE OF HIV-1-INFECTED CELLS IN
BREASTMILK AT DAY 15

PCR	Child's HIV-1 status		
	Infected	Not infected	Indeterminate
Positive (n = 60)	19 (32%)	32 (53%)	9 (15%)
Negative (n = 69)	11 (16%)	51 (74%)	7 (10%)
Total (n = 129)	30 (23%)	83 (64%)	16 (13%)

TABLE IV—MULTIVARIATE ANALYSIS OF POTENTIAL RISK
FACTORS FOR MOTHER-TO-CHILD TRANSMISSION OF HIV-1

—	p	Odds ratio (95% CI)
Positive PCR in day 15 milk sample	0·01	5·40 (1·40–20·0)
CD4/CD8 ratio <0·5 on day 15 maternal blood sample	0·07	3·45 (0·90–12·5)
HIV-1 IgM in breastmilk persisting up to 18 months	0·05	0·11 (0·01–1·02)

cells for PCR. However, there was no difference in CD4/CD8 ratio between mothers whose milk was or was not tested by PCR at day 15 (mean 0·77 [SD 0·49] vs 0·82 [0·56], p = 0·57). Similarly, there was no difference in distribution of HIV-1 infection status between children born to mothers with and without PCR done on milk 15 days post partum (χ^2 test, p = 0·81).

At each collection time, the most frequently detected immunoglobulin class for specific anti-HIV-1 in breastmilk samples was IgG (figure). IgM was the next most common, and IgA was found less often. The frequency of a positive PCR declined from 47% at 15 days to 21% at 6 months (MacNemar test for 65 paired samples, p < 0·01).

The detection of specific IgM in milk samples 15 days post partum was not related to the immune status of the mothers. The mean CD4/CD8 ratio in 105 mothers with milk samples positive for HIV-1 IgM was 0·78 (0·52) compared with 0·70 (0·43) in 48 mothers with milk samples negative for HIV-1 IgM (Student's t test, p = 0·36). Nor was the detection of HIV-1 IgA at 15 days associated with the mother's immune status (CD4/CD8 = 0·82 [0·52] vs 0·73 [0·43] in 35 IgA-positive and 115 IgA-negative mothers, respectively; p = 0·38). Similarly, we found no relation between the likelihood of HIV-1 infection in the children and the detection of HIV-1 IgM or IgA at any collection time. Among 115 children fed with breastmilk containing HIV-1 IgM at day 15, 23 (20%) were HIV-1 infected, 75 (65%) not infected, and 17 (15%) of indeterminate status compared with 14 (26%), 37 (70%), and 2 (4%), respectively, among the 53 children fed with breastmilk not containing IgM (Mantel-Haenszel χ^2 adjusted for mother's immune status, p = 0·68; Mantel-Haenszel weighted odds ratio 0·76 [0·31–1·91]).

We assessed the relation between HIV-1 infection status of the children and the number of milk samples positive for HIV-1 IgM and IgA by means of a trend analysis. By definition, this analysis could include only the 84 children who lived beyond the age of 18 months and whose mothers provided the laboratory with all three milk samples. The persistence of milk HIV-1 IgM antibodies was linearly associated with the absence of HIV-1 infection in children (χ^2 for trend, adjusted for mother's immune status = 6·66, p = 0·01, table II). There was a similar, though weaker, association for the persistence of milk HIV-1 IgA ($\chi^2 = 3·79$, p = 0·05).

All 34 milk samples from HIV-1-seronegative mothers used as internal controls remained consistently unreactive by PCR. The detection of HIV-1-infected cells in the milk samples 15 days post partum was related to the immune status of the seropositive mothers; the mean CD4/CD8 ratio was 0·66 (0·43) in mothers with PCR-positive milk samples and 0·87 (0·53) in those with PCR-negative samples (Student's t test, p = 0·03). The detection of integrated DNA in milk cells 15 days post partum was independent of the presence of HIV-1 IgA or IgM in milk (HIV-1 IgA and PCR, p = 0·34; IgM and PCR, p = 0·38, χ^2 test). The detection by PCR of infected milk cells at 15 days was associated with the subsequent development of HIV-1 infection in the infants (odds ratio = 2·75 [95% CI 1·07–7·16]; table III). Adjustment for mother's immune status and IgM results did not affect this association. By contrast, no association with HIV-1 infection in the child was found for PCR on milk samples collected at 6 months (p = 0·21).

Multivariate analysis was done on data for 63 children who survived more than 18 months (17 HIV-1 infected and 46 not infected). A positive PCR in the milk sample collected 15 days post partum was the only factor significantly associated with transmission of HIV-1 from mother to infant (table IV). The relation of an inconsistent or non-persistent IgM anti-HIV-1 response in milk with transmission was of borderline significance.

To verify our hypothesis that some infants could have been infected by ingestion of milk containing HIV-1-infected cells but with a defective IgM anti-HIV-1 response, the distribution of infants' HIV-1 status was examined according to the presence of infected cells in 15-day milk samples with or without HIV-1 IgM. This trend analysis was consistent with the proposed hypothesis, even after control for the mother's immune status (adjusted χ^2 for trend = 6·28, p = 0·01, table v). The association was not affected by use of extreme assumptions; if all 14 children of indeterminate HIV-1 infection status were taken as not infected p is 0·01, and if they are all taken as infected p is 0·02. Thus, in children fed with early breastmilk containing HIV-1-infected cells but lacking specific IgM antibodies, the likelihood of being infected was 47%, which was significantly higher than that for children fed breastmilk without HIV-1-infected cells (18%). In the latter subset of children, the presence or absence of specific IgM did not affect the risk of HIV-1 acquisition (4 infected children of 20

TABLE V—INTERACTIONS BETWEEN HIV-1 IgM AND HIV-1-INFECTED CELLS DETECTED IN MILK SAMPLES AT DAY 15 AND LIKELIHOOD
OF HIV-1 INFECTION IN CHILDREN

Test results on milk samples collected at day 15		Child's HIV-1 status			Transmission rate (%) (95% CI)*	Mantel-Haenszel weighted odds ratio (95% CI)*
PCR	IgM	Infected	Not infected	Indeterminate		
Negative	Positive or negative	11	51	7	18 (8–28)	1·00
Positive	Positive	8	19	6	30 (13–47)	2·35 (0·66–8·34)
Positive	Negative	9	10	1	47 (22–69)	4·51 (1·09–18·96)
All	All	28	80	14	26 (18–34)	

*Transmission rates and odds ratios were calculated by excluding children with indeterminate HIV-1 infectious status and adjusting for mother's CD4/CD8 ratio.

fed PCR-negative/IgM-negative milk vs 7 of 42 fed PCR-negative/IgM-positive milk; two-tailed Fisher's exact test, p = 0·74; odds ratio = 1·25 [0·26-5·79]).

Discussion

This study describes the frequency and the timing of specific humoral responses against HIV-1 in the milk of infected mothers and the relations to maternal-child transmission of HIV-1. Although it is based on qualitative measurements, several characteristics of the specific biological properties of breastmilk against HIV-1 can be inferred. Secretory IgA antibodies are the main component of humoral immunity in breastmilk.[17] They probably protect against viral disease by coating and blocking viral attachment and by neutralisation of viral particles.[18] By contrast with many other infectious diseases, specific HIV-1 IgA in breastmilk was rarely detected in this study. Other body secretions, such as saliva[19] and seminal fluid[20] from HIV-infected subjects contain smaller amounts of HIV-1-specific and non-specific IgA than expected, which may reflect impairment of mucosal immunity. Usually colostrum and early breastmilk contain large amounts of IgA, which steadily decline during the first few months of lactation. By contrast in our study, there was a progressive increase in the frequency of HIV-1 IgA. Defective synthesis of secretory IgA can be compensated for by secretory IgM (IgM antibodies with a secretory component).[21] Indeed, in many cases we found sustained humoral responses against HIV-1 of the IgM type. We did not test for HIV-1 IgA and IgM in the blood samples of our HIV-1-infected mothers. However, in a previous study, these antibodies were not detected in the serum samples of mothers with milk samples positive for IgA, IgM, or both; this finding suggests that most of the IgA and IgM antibodies detected in milk are synthesised within the mammary gland.[22]

Our study was limited by a major methodological difficulty. Because there are no diagnostic tools to determine HIV infection status of newborn infants with an acceptable level of confidence, we could not distinguish in-utero infections from those acquired during or after birth. Consequently, we had to refer to an overall risk of transmission of HIV from mother to child.

HIV-1-infected cells were found by double PCR in many breastmilk samples from HIV-1-infected mothers collected early in the lactation period. The frequency of detection declined with the duration of lactation, which is consistent with the striking decline in the cell contents of breastmilk during the first 8 weeks of lactation.[23] We should emphasise that integrated segments of viral DNA detected by PCR do not necessarily represent infectious virus, since defective integrated virus could be identified by this extremely sensitive method. We found PCR-positive breastmilk samples more frequently in mothers with severe immune deficiency than in those who were immunocompetent. However, the presence of HIV-1-infected cells in early breastmilk was associated with HIV-1 infection in the child, independently of the mother's immune status. By multivariate analysis, the presence of infected cells in breastmilk was the factor most strongly associated with HIV-1 transmission to children of HIV-1-infected mothers who survived more than 18 months. This factor was even more strongly associated with transmission than the mother's immune status, a variable already shown to be related to transmission when applied to the entire cohort in the same study[12] and in others.[11] Two smaller studies[24,25] have found associations between the detection of integrated

viral DNA in cells from breastmilk and mother-to-child transmission of the virus.[24,25] Such observations are consistent with the postnatal transmission of many human and animal retroviruses, which can be passed from mother to offspring by infected macrophages and lymphocytes in milk.[1] For bovine leukaemia virus, as few as 2000 infected cells can transmit the infection.[26] Our finding of no association between the presence of infected milk cells in 6-month samples and mother-to-infant transmission suggests that most of the cases of postnatal transmission through breastfeeding occur early in lactation if the mother has been infected before delivery.

Our study suggests that HIV-1 IgM in breastmilk could be protective against postnatal transmission of the virus. There are several possible explanations of this effect. First, secretory IgM could compensate for a defective secretory IgA response and behave in a similar way by directly coating viral particles. Second, IgM antibodies are strong potentiators of complement-mediated cytotoxicity because of their high content of Fc sites. At least nine components of the complement system have been identified in human milk.[17] These components could have powerful lytic properties against HIV-1 virion[27] and, perhaps, against HIV-1-infected cells in association with specific IgM.[28] Third, as shown for murine sarcoma virus, specific IgM could take part in the lysis of infected cells by a mechanism of antibody-dependent lymphocyte cytotoxicity.[29]

We did not aim to quantify the risk of transmission of HIV-1 through breastfeeding nor to find out when HIV infection occurred. Indeed, some of the children who were assumed to have been infected postnatally could well have been infected before birth. Our study suggests that some biological factors in milk are associated with mother-to-child transmission and that others seem to protect against infection. The consistency of our biological model, the strength of the associations documented, and their biological plausibility suggest that these factors are indeed determinants of transmission and not intermediate or confounding variables. The results of this study should not be interpreted as a promotion of formula feeding. The recent recommendations of the WHO and UNICEF[30] on the promotion of breastfeeding remain valid and are certainly not questioned by our findings.

In our cohort, children fed with early breastmilk without HIV-1-infected cells had a low risk of acquisition of HIV-1 from their mothers. In these children, the rate of transmission is similar to that estimated in infants in countries where formula feeding by HIV-infected mothers is almost the rule.[4,5] On the other hand, children fed with breastmilk containing HIV-1-infected cells but not HIV-1-specific IgM had a very high risk of infection (47%). Our study seems to validate a model for the pathogenesis of HIV-1 acquisition by breastfeeding in which infected cells confer a risk of transmission that could be circumvented by the local production of specific IgM with cytotoxic properties, neutralising properties, or both. Our findings indirectly suggest that a vaccine preparation inducing a persistent immune response of the IgM type in the mother's body fluids could be valuable to prevent transmission of HIV-1 from mother to child. Such hypotheses require confirmation before specific interventions are developed and tested in populations with greatest need, namely women living in developing countries with high prevalence and incidence of HIV infection.

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From The Lancet

Giving a specimen

Expert seminologists in this country are still scarce, and their knowledge has so far not permeated the vast bulk of clinicians and pathologists called in to report on the fertilising power of a given specimen of semen. It is not sufficient to glance down a microscope and report no sperms, sperms in plenty, mobile sperms, or sperms dead: in many cases these are false readings and the patient is misdiagnosed, often with serious consequences to marital equilibrium. The examination of the male in a case of sterility consists of a careful clinical survey . . . . The patient's general physical condition and development is then assessed, constitutional and metabolic disorders being eliminated or investigated . . . . The next step is the urological examination of the external genitalia, prostate and vesicles, and the exclusion of congenital and acquired defects . . . . This is followed by the most important single investigation, the examination of a specimen of semen. Semen can be collected by two methods, masturbation or condom, and in each certain details must be observed or the specimen is vitiated. Both have inherent defects, and some men find considerable difficulty in obtaining either, but these obstacles can be overcome by the tactful cooperation of the doctor. A masturbation specimen must be collected in a clean dry wide-necked glass receptacle with a stopper. The glass must be clean, and for preference old and much used since new glass reacts injuriously on spermatozoa; it must be dry because hypotonic solutions affect sperm viability. Such a specimen can be collected in or near the laboratory and be examined at once, and this is an advantage, but some nervous men find this method distasteful or impossible, and for them the condom must be used. An ordinary rubber condom is satisfactory provided it is thoroughly washed in running water and then dried on clean filter paper; the outside only should be powdered before re-rolling. Continence should be enjoined for a week before the production of the specimen, which should be obtained by ordinary coitus with as short a delay as possible before it is examined. After coitus it is best to transfer the contents of the condom to a glass container, and this is best done by snipping the end of the condom with scissors. If transport takes time the glass container should be kept cool—in a thermos flask of cold tap water—never warm, so it should not be put in that handy human incubator the waistcoat pocket. All these trivial details are important and can be best secured by issuing each patient with a small typescript card of instruction.

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