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Limiting dilution analysis of the allo-MHC anti-paternal cytotoxic T cell response I: normal primigravid and multiparous pregnancies

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SUMMARY

Anti-paternal cytotoxic T lymphocyte precursor frequencies (CTLpF) were determined by limiting dilution analysis (LDA) in the peripheral blood of eight primigravid and seven multiparous women during the three trimesters of pregnancy. In five of these women the responses to cord blood lymphocytes (CBL) and paternal lymphocytes were also determined at the time of delivery and at 6 weeks post delivery. As controls, CTLpF against unrelated third party donors were determined. A wide range of CTLpF against all three groups of targets was found in both the primigravid and multiparous women, reflecting the wide range of frequencies found in random populations. These frequencies remained fairly constant during and 6 weeks after the pregnancy. Splitwell analysis demonstrated that the responses generated in our culture system were specific to the stimulator. The LDA data conform to single-hit kinetics, indicating that only cytotoxic T cells were limiting in the assay. Proliferative responses of maternal lymphocytes to paternal, cord blood and third party MHC antigens also remained unchanged as determined by time-course mixed lymphocyte reactions (MLR). Our data suggest that there is no significant allo-stimulation or suppression of the maternal immune system during normal pregnancy. The mother remains immunocompetent and is capable of both cytotoxic and proliferative responses to paternally-derived fetal MHC antigens. Our findings confirm that in normal pregnancy the trophoblast, which is devoid of classical MHC antigens, forms an effective immune barrier which prevents interaction of the maternal and fetal immune systems.

Keywords limiting dilution analysis cytotoxic T lymphocyte precursor allo-MHC primigravid multiparous

INTRODUCTION

The success of viviparous pregnancies in vertebrates has posed a fundamental paradox since the discovery of the laws of transplantation immunology in the late 1940s. The pregnant female might be expected to mount an immune response against her semi-allogeneic fetus, yet no evidence of graft rejection is seen and the pregnancy proceeds unimpeded. Many hypotheses have been advanced to explain the mechanism of success of what has often been referred to as nature's perfect allograft. These include (i) the existence of an immunologically inert barrier between maternal and fetal cells [1,2], (ii) immunosuppressive environmental factors [3], and (iii) expression of regulatory proteins that interfere with the complement cascade [4]. Over the years strong evidence has accumulated for all three.

In studies on human pregnancies the phenomenon of otherwise unexplained recurrent spontaneous abortion (RSA) has often been regarded as having an immunological basis. The basic assumption has been that RSA represented a failure of some ill-defined immunoregulatory mechanisms. Rocklin *et al.* [5] reported that women with RSA lacked a 'blocking antibody' which they postulated shielded paternally-derived fetal HLA antigens. Subsequent reports of increased HLA antigen sharing among couples with RSA [6,7] led to the hypothesis that an important requirement for successful pregnancy was a significant MHC disparity between the mother and father, which allowed the maternal immune system to recognize the foreign fetal HLA antigens, thus mounting a protective response. Animal models provided ample evidence for this: for example, in mouse strain combinations with a high frequency of intrauterine resorption, resorption can be reduced to a more normal frequency by a prior pregnancy from a male of the same paternal strain, by immunization with lymphocytes from the paternal strain, or by passive transfer of immune IgG [8]. Thus it has been supposed that the mother makes some response to paternally-derived MHC antigens.

Maternal antibodies to paternally-derived fetal HLA are common in the sera of multiparous women [9], but a recent systematic study in primigravid pregnancies showed that these

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antibodies do not occur in the majority of primigravid pregnancies, and that when they do occur they do so in late pregnancy [10]. It has been difficult to demonstrate cytotoxic T lymphocytes (CTL) specific for paternal HLA: reports addressing this issue tended to employ semiquantitative and rather insensitive techniques such as cell-mediated lympholysis [11]. Yet the issue of whether anti-paternal CTL are generated in pregnancy is central to the question of the success of pregnancy: any rejection response might be assumed to involve CTL. We have therefore carried out a detailed analysis of the maternal allo-response in normal primigravid and multiparous pregnancy. We have used limiting dilution analysis (LDA) to study the anti-paternal cytotoxic T cell response throughout normal pregnancy, at delivery and 6 weeks after delivery. LDA is a powerful means of estimating the frequencies of cells participating in the immune response, and it is now the standard tool for estimating frequencies of cytotoxic T precursor cells [12,13]. The quantitative capacity, reproducibility and specificity of LDA makes it a useful tool for clinical applications, such that it has been used in follow-up studies of host anti-graft reactivity in renal transplantation [14–16], and has also been used as a predictor of graft-*versus*-host disease in bone marrow transplantation [17]. Indeed, van Rood & Claas have gone as far as hailing LDA as possibly the 'Holy Grail' in transplantation: an *in vitro* test to predict graft outcome [18]. A multi-centre European workshop is currently studying the possibility of standardizing LDA protocols in organ transplantation (Sharrock and Claas, personal communication).

PATIENTS AND METHODS

Patients

Eleven healthy primigravid and seven multiparous (minimum three previous uncomplicated pregnancies) women and their partners were recruited from our booking clinic. Peripheral blood samples were obtained from these women during the first trimester (8–10 weeks), second trimester (24–28 weeks), third trimester (34–38 weeks), in five of them on the day of delivery, when cord blood was also taken, and at 6 weeks after delivery. Both husband and wife, and where appropriate cord blood, were HLA tissue-typed by conventional cytotoxicity assay. None of the women had anti-paternal cytotoxic antibodies (APCA).

Preparation of cells

Peripheral blood lymphocytes (PBL) from each donor were prepared on a lymphoprep density gradient (Nycomed As, Oslo, Norway) and stored in liquid nitrogen for subsequent simultaneous analysis. Immediately before setting up the assays, cells were thawed rapidly, washed twice in culture medium, and resuspended at the desired concentration. All assays for each woman were carried out on the same day.

Tissue culture conditions

The tissue culture medium (TCM) used in all experiments was RPMI 1640 supplemented with 5% heat-inactivated pooled AB serum (Blood Transfusion Service, South West Thames Region, UK), 25 mM L-glutamine, penicillin 50 U/ml and streptomycin 50 mg/ml (GIBCO, Paisley, UK). Cells were cultured in 96-well round-bottomed microtitre plates (Costar, UK) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Limiting dilution cultures

Limiting dilution (LD) cultures were set up in 96-well round-bottomed microtitre plates as previously described [12,13]. Briefly, limiting numbers of responder (wife's) cells (5×10^4–312 per well) were co-cultured with a constant number (5×10^4) of irradiated (30 Gy) stimulator cells from the husband or cord blood in one set of experiments and from a third party in a parallel set. Total culture volume was 150 μl/well, and there were 24 replicates for each responder cell concentration. The LD cultures were incubated for 10 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. On days 3 and 7 the cultures were fed with 50 μl of fresh culture medium supplemented with recombinant IL-2 to achieve a final concentration of 10 U/ml in the culture wells.

Preparation of PHA blasts. On day 7, previously cryopreserved peripheral blood mononuclear cells (PBMC) from the husband, cord blood and third party were thawed, washed and resuspended at 1×10^6/ml in TCM containing 2 μg/ml phytohaemagglutinin (PHA; Sigma, Poole, UK). Ten millilitres of each cell suspension were cultured in an upright 25-ml Falcon tissue culture flask (Becton Dickinson, UK) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cytotoxicity assay. On day 10 the PHA blasts were washed and labelled for 90 min with ^{51}Cr (100 μCi/10^7 blasts; sodium chromate CJS.4, diluted to 3 mCi in 0·9% NaCl, Amersham, Amersham, UK) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The blasts were washed three times and then resuspended at 1×10^5 cells/ml in TCM before use. One hundred microlitres of supernatant were removed from each well of the LD culture plates. The cell pellets in the LD cultures were homogenized by shaking the plates on a micro-shaker (Dynatech). One hundred microlitres of the PHA blasts were added to each well (10^4 blasts/well). The plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The plates were then centrifuged at 400 ***g*** for 30 s. One hundred microlitres of supernatant were harvested from each well for gamma-ray counting. Total ^{51}Cr release was assessed by incubating labelled blasts with a 1% solution of Triton-X in distilled water, and spontaneous release by incubating the blasts in TCM.

Splitwell analysis

To analyse the fine specificity of the cytotoxic T cells generated in the LD cultures, each individual well from the LD cultures was split into two. One half was assayed for lytic activity against target PHA blasts derived from the original stimulator cell donor, and the other half against blasts from a third party not used as a stimulator in the LD culture. For example, maternal cytotoxic cells generated by stimulation with the husband's lymphocyte were tested for lytic activity against PHA blasts derived from the husband as well as those derived from third party or cord lymphocytes. Likewise, maternal cytotoxic cells generated in response to third party lymphocytes were tested for lytic activity against the third party PHA blasts as well as the husband's PHA blasts.

Statistical analysis

For LD assays, wells were scored positive if their ^{51}Cr release was greater than the ^{51}Cr release (mean + 3 s.d.) observed in 24 replicate microwells containing irradiated stimulator cells and PHA blasts, but no responder cells. The frequency of responding cells was calculated by maximum likelihood estimation

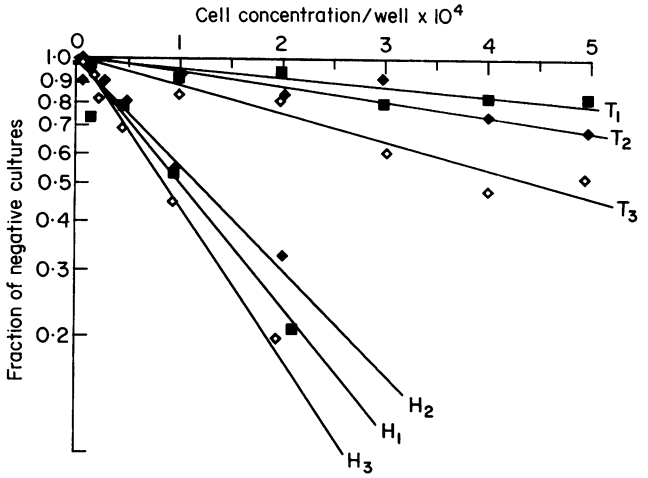

Fig. 1. Example of cytotoxic T lymphocyte precursor (CTLp) frequencies against husband (H) and third party (T) MHC antigens in the first (H_1, T_1), second (H_2, T_2) and third (H_3, T_3) trimesters of pregnancy. The 95% confidence limits for the frequencies against each target in all three trimesters overlapped, indicating that the frequencies at the three gestations were not significantly different.

using a computer program which also calculated the 95% confidence intervals of the data and the χ^2 estimate of probability. Regression analysis was used to present the data graphically and χ^2 analysis was used to show that the data conformed to single-hit kinetics.

Time-course mixed lymphocyte reactions

Quintuplicate 200-μl cultures (2×10^5 cells/well) were set up in 96-well round-bottomed microtitre plates containing the wife's cells as responders and stimulator cells from the husband or third party in a ratio of 1 : 1. Stimulator cells were inactivated by irradiation (30 Gy). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Proliferation was measured daily (separate plates) from day 1 to day 6: 1·0 μCi ^{3}H-thymidine (TRK 120, 25 Ci/mmol, Amersham) was added to each well, and after 18 h further incubation the cultures were harvested onto glass fibre filters using a cell harvester (Skatron, Lier, Norway) and the radioactivity was counted by liquid scintillation. Results for each experiment were expressed as the mean ct/min of the quintuplicate cultures.

RESULTS

Frequencies of cytotoxic T lymphocyte precursors

Three of the 11 primigravid women were excluded from our analysis because their pregnancies subsequently developed complications. The rest of the women therefore had apparently uncomplicated pregnancies, culminating in the normal vaginal delivery of healthy infants.

Cytotoxic T lymphocyte precursor frequencies (CTLpF) against husband and third party MHC antigens were determined in each trimester of pregnancy. In addition, in five of these women CTLpF were also determined against cord blood lymphocytes (CBL) at delivery and at 6 weeks post-partum. The results of a typical experiment are depicted in Fig. 1: the regression lines fitted the Poisson distribution, with $P > 0{\cdot}05$ for linearity, with all the lines passing through the origin, thus the frequencies obtained were consistent with a single-hit kinetic model, indicating that only cytotoxic cells were limiting in our assay system. By convention in LDA, when the 95% confidence intervals of two frequencies overlap, then any difference between the two frequencies is considered insignificant.

A summary of the CTLpF against paternal and third party MHC antigens is given in Table 1. Our data show that generally for each woman there was no significant change in the CTLpF throughout and after pregnancy. There were three exceptions (cases 2, 6, and 7, Table 1), but even in these at least two CTLpF were similar. There was a wide range of CTLpF against both paternal and third party MHC antigens.

The CTLpF against fetal MHC antigens are shown in Table 2. Again, no significant change was seen in the responses of each woman at the time points assessed, and a wide range of frequencies is observed.

Fine specificity of the CTL: splitwell analysis

To determine the specificity of the individual clones generated in the LD cultures, splitwell analysis was used. Responder (wife) cells were cultured with irradiated allogeneic (partner, CBL or third party) stimulator cells in a standard LDA culture. On day 10 each well in the culture was divided into two equal portions in fresh plates. One half was tested for cytotoxicity against PHA blasts from the original stimulator, and the other half against HLA mismatched third party PHA blasts. Chromium release from one half was plotted against release from its corresponding half. In Fig. 2, the dot plots obtained from one typical experiment illustrate the specificity of the clones. In Fig. 2a, only three clones generated in response to paternal MHC antigens lysed third-party targets, while the rest did not; and likewise clones generated against third-party MHC antigens did not lyse paternal cells (Fig. 2b). Clones generated in response to paternal antigens lysed CBL (Fig. 2c), and the converse was true (Fig. 2d). Interestingly, clones generated in response to CBL were more efficient at lysing paternally-derived targets than CBL-derived targets (Fig. 2d), and this trend was observed in all five experiments.

Time-course MLR

We used time-course MLR to study the kinetics of the proliferative responses of pregnant women to paternal, cord blood and third party MHC antigens. No significant changes in the kinetics of the MLR were observed for each woman throughout and after pregnancy (Fig. 3a–d).

DISCUSSION

We have described the use of LDA and time-course mixed lymphocyte cultures to evaluate the maternal immune response to paternal and fetal MHC antigens during the course of apparently normal pregnancies in both primigravid and multiparous women. Before the definitive experiments described in this study, we had taken care to optimize our limiting dilution culture systems (unpublished data): we had established the reproducibility of the assay, and validated the use of splitwell analysis to study the fine specificity of the clones generated in our cultures. We had also established that the use of cryopreserved lymphocytes did not significantly affect our results. Thus in all the experiments we used previously cryopreserved cells, making it possible to set up the full set of experiments for a given

Table 1. Limiting dilution analysis (LDA) of the frequency of husband-reactive (H) and third party-reactive (T) cytotoxic T lymphocyte precursors (CTLp) in women during the three trimesters of pregnancy (cases 1–8, primigravid women; cases 9–15, multiparous women)

		1st trimester		2nd trimester		3rd trimester	
Case	Target	1/f	95% CL	1/f	95% CL	1/f	95% CL
1	H	1/30 318	25 320–43 148	1/34 440	29 889–48 573	1/27 971	24 245–38 440
	T	1/4017	3207–5438	1/4914	3318–6194	1/6651	3517–8181
2	H	1/6767	4399–8243	1/10 510	8100–13 321	1/14 740	11 010–17 966
	T	1/20 684	15 904–30 299	1/25 110	21 874–34 171	1/21 392	17 876–25 981
3	H	1/29 719	21 865–40 329	1/23 865	17 848–31 909	1/35 491	20 200–62 358
	T	1/5978	4379–7739	1/6180	4199–7498	1/8001	5233–11 281
4	H	1/13 720	10 507–17 915	1/13 019	9981–16 981	1/14 290	10 933–18 677
	T	1/45 840	36 308–89 129	1/55 782	41 732–74 480	1/60 313	49 157–75 392
5	H	1/10 808	7314–15 617	ND	—	1/13 430	10 320–16 741
	T	1/5818	7379–13 898	ND	—	1/10 101	8278–13 154
6	H	1/21 115	15 858–28 115	1/38 469	29 027–50 982	1/19 567	14 657–26 121
	T	1/9882	7353–13 919	1/7718	53 232–10 187	1/10 240	8117–12 918
7	H	1/17 658	13 782–22 623	1/25 390	19 496–33 067	1/13 140	10 058–17 166
	T	1/13 289	9077–17 427	1/16 847	13 169–19 047	1/16 147	12 006–18 188
8	H	1/82 252	78 477–87 612	1/79 014	76 444–84 189	1/82 799	79 457–85 384
	T	1/24 766	16 918–32 934	1/19 187	14 668–23 458	1/21 332	18 465–26 189
9	H	1/41 892	32 503–53 992	1/31 560	24 117–41 299	1/36 084	29 441–46 128
	T	1/15 135	10 514–19 757	1/15 718	11 618–19 748	1/15 224	10 556–18 764
10	H	1/3318	2532–4348	1/4526	3464–5912	1/3225	2460–4227
	T	1/16 545	11 455–21 764	1/13 946	9914–17 841	1/10 415	8670–14 534
11	H	1/50 147	39 381–56 704	1/58 721	42 425–67 874	1/58 108	41 119–64 212
	T	1/9282	7332–13 904	1/12 384	8018–16 199	1/14 244	11 394–18 375
12	H	1/6034	4354–7919	1/4625	3644–5108	1/5209	4722–7206
	T	1/39 947	34 323–46 127	1/42 238	34 921–51 920	1/48 122	38 728–58 720
13	H	1/34 348	28 701–40 011	1/36 270	31 041–44 417	1/30 800	24 818–37 194
	T	1/2472	1947–4390	1/4262	2214–6119	1/5128	2799–7812
14	H	1/57 824	45 460–80 001	1/62 764	50 112–91 464	1/58 824	48 460–83 478
	T	1/62 274	55 889–70 011	1/54 641	45 280–62 188	1/50 110	41 888–62 941
15	H	1/6402	6124–10 706	1/6114	4328–7538	1/7720	5968–9294
	T	1/24 800	17 918–29 394	1/19 284	16 275–24 118	1/21 274	17 284–26 766

Table 2. Limiting dilution analysis (LDA) of the frequency of cord blood lymphocyte (CBL)-reactive (CB) and husband-reactive (H) cytotoxic T lymphocyte precursors (CTLp) in women during the three trimesters of pregnancy, at delivery and 6 weeks post-delivery.

		1st trimester		2nd trimester		3rd trimester		At delivery		Six weeks post-delivery	
Case	T	1/f	95% CL	1/f	95% CL	1/f	95% CL	1/f	95% CL	1/f	95% CL
1	CB	1/39 497	33 264–53 519	1/34 004	29 407–41 070	1/36 308	31 192–44 192	1/42 374	34 141–50 918	1/38 814	31 911–49 727
	H	1/30 318	25 320–43 148	1/34 440	29 889–48 573	1/27 971	24 245–38 440	1/35 040	30 947–41 801	1/29 485	21 844–34 321
5	CB	1/12 601	11 992–14 100	ND	—	1/12 798	11 874–13 871	1/14 144	12 346–11 166	1/13 658	12 394–15 103
	H	1/10 808	7314–15 617	ND		1/13 430	10 320–16 741	1/11 640	8451–15 827	1/14 728	11 986–16 287
6	CB	1/41 505	34 744–49 164	1/50 382	42 821–56 949	1/37 154	29 189–44 718	1/39 128	33 818–50 110	1/48 347	37 376–55 317
	H	1/21 115	15 858–28 115	1/38 469	29 027–50 982	1/19 567	14 657–26 121	1/24 268	18 612–28 982	1/22 104	19 675–26 349
10	CB	1/3526	2693–4618	1/4815	2901–6104	1/3685	2264–4814	1/4128	3100–6421	1/3018	2662–4285
	H	1/3318	2532–4348	1/4526	3464–5912	1/3225	2460–4227	1/4002	2506–6312	1/4772	2940–6267
15	CB	1/10 284	8187–13 154	1/14 544	11 874–16 194	1/8149	6794–11 274	1/11 784	9144–14 821	1/16 948	12 338–19 147
	H	1/6402	6124–10 706	1/6114	4328–7538	1/7720	5968–9294	1/6801	4970–9418	1/7298	6497–11 472

Cases 1, 5 and 6, primigravid women; cases 10 and 15, multiparous women; T, target.

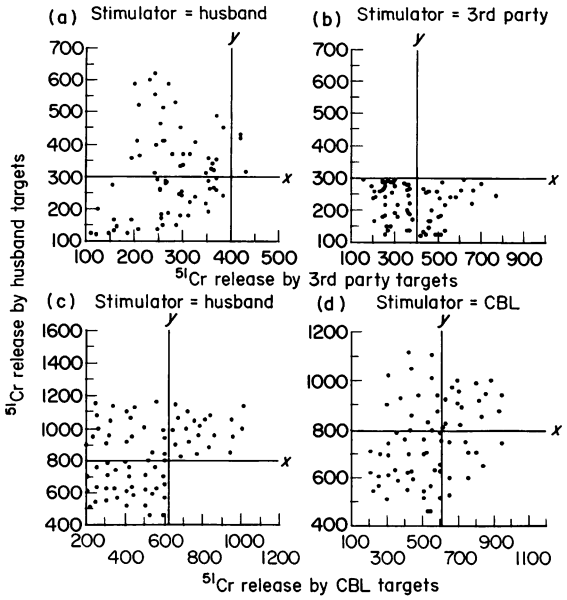

Fig. 2. Fine specificity of the cytotoxic T lymphocyte (CTL): to determine the specificity of the individual clones generated in the limiting dilution (LD) cultures, splitwell analysis was used. Responder (wife) cells were cultured with irradiated allogeneic (partner, cord blood lymphocyte (CBL) or third party) stimulator cells in a standard LDA culture. On day 10, each well in the culture was divided into two equal portions in fresh plates. One half was tested for cytotoxicity against phytohaemagglutinin (PHA) blasts from the original stimulator, and the other half against non-stimulator PHA blasts. Chromium release from one half was plotted against release from its corresponding half to produce a dot plot. For clarity, chromium release at three cell concentrations only has been plotted. The lines x and y are the mean + 3 s.d. chromium release from control targets, and therefore represent the cut-off between positive and negative wells: values above the line x or to the right of y represent positive wells.

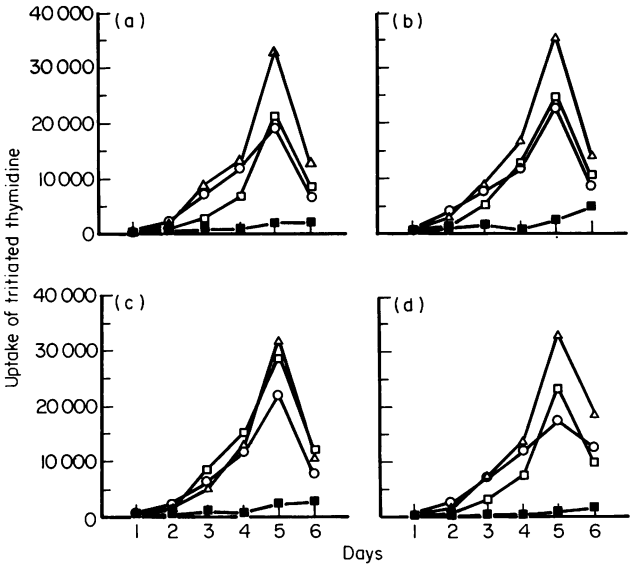

Fig. 3. Time-course MLR: the kinetics of the proliferative responses of pregnant women to paternal (□), cord blood (○), third party (△) and self (■) MHC antigens were studied in MLR cultures in the three trimesters (a, 1st trimester; b, 2nd trimester; c, 3rd trimester) and 6 weeks post-delivery (d) in five women (cases 1, 5, 6, 10 and 15). Proliferative responses were measured daily from day 1 to day 6. The arithmetic means of the responses of the five women are shown, and for clarity the error bars have been omitted.

patient at the same time. For the time-course MLR, culture plates were pulsed with tritiated thymidine for the appropriate periods and the plates were then stored at −20°C, to be harvested and assessed for radioactive uptake once the full assay had been completed. In this way we minimized variation due to changing experimental conditions.

We studied primigravid and multigravid women on the assumption that responses in the latter might be influenced by previous pregnancies, and we recruited only those multiparous women who had had at least three normal pregnancies. In cytotoxic responses to paternal, fetal and third party MHC antigens, we found a wide range of frequencies of CTL in both groups of women, and these frequencies did not change significantly throughout the three trimesters, at delivery and 6 weeks post-delivery. In time-course MLR, we did not observe any difference in the kinetics of the proliferative responses between primigravid and multiparous women. The latter might have been expected to exhibit a secondary-type response to paternal and/or fetal MHC antigens if they had been primed during previous pregnancies. No change was observed in both groups throughout and after pregnancy, and Fig. 3 depicts the arithmetic means of the time course MLR of five women who were studied during and after pregnancy (cases 1, 5, 6, 10 and 15). Our data thus show that the human female remains immunocompetent throughout pregnancy, being capable of mounting cytotoxic as well as proliferative allo-responses to her fetus as well as a third party. That there is no evidence of a rejection response suggests that no allo-stimulation occurs. The trophoblast, which lacks classical MHC antigens, and which comes into direct contact with the maternal immune system, forms an effective immunologically inert barrier. Lack of any significant qualitative and quantitative change in the immune responses as demonstrated by our data supports this idea.

The wide range of frequencies of CTLp we found is consistent with previous reports of studies of frequencies of alloreactive CTLp in random populations [19]. The molecular basis of allo-recognition is poorly understood, but is thought to be based on cross-reactivity by antigen-specific, self-MHC restricted T cells [20,21]. Thus the finding of a wide range of frequencies of CTLp is to be expected, and is consistent with the hypothesis of cross-reactivity. Responses to allo-MHC antigens would be influenced by, among other factors, the extent of disparity between the responder and stimulator: thus responses to CBL would tend to be less than those to paternal or third party antigens, since the fetus is haplo-identical to the mother. Using splitwell analysis, we demonstrated that CTL clones generated in response to paternal MHC antigens did not lyse third party cells and *vice versa*. Because of shared target antigens, it was not surprising to find that clones generated in response to paternal MHC antigens could lyse CBL. It was interesting that clones generated in response to fetal MHC antigens were often more efficient at lysing paternally derived targets than CBL-derived targets (Fig. 2d). We are unable to explain this, but we speculate that this may be due simply to differences in epitope density; it is also conceivable that the presence of maternally derived MHC antigens alongside pater-

nally derived ones on the CBL blasts influences the ability of maternal CTL clones to lyse them.

We conclude that in humans, contrary to other reports, there is an intact cellular immune response against paternal MHC antigens, and despite this the fetus survives. Lack of priming suggests that the MHC antigen-deficient trophoblast forms an effective barrier. Other observed phenomena such as immunosuppressive factors [3] and regulatory proteins that interfere with the complement cascade [4] may act synergistically to protect the fetus, but the trophoblast barrier would seem to be the most important factor in the survival of the fetal semi-allograft.

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