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Villitis of Unknown Etiology Is Associated with Major Infiltration of Fetal Tissue by Maternal Inflammatory Cells

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Villitis of unknown etiology (VUE) is a common placental lesion and an important cause of intrauterine growth retardation and recurrent reproductive failure. Two theories have been proposed to explain VUE. One proposes that VUE is an exclusively fetal immune response to microbial antigen, whereas the other suggests that maternal cells cross the maternal-fetal interface and mount an immune response to fetal antigens. To differentiate between these alternatives, we performed* in situ *hybridization using X and Y chromosome-specific probes and immunostaining for CD3 and CD45 on VUE placentas from male infants. A total of eight foci and 40 villi were studied from four male VUE placentas. Controls included nonaffected villi from each male VUE placenta, a female VUE placenta, and male and female tonsils. Affected villi showed a major proportion of XX (maternal) cells (range 30 to 54%). An appropriate percentage of the remaining (fetal) cells contained Y chromosomes. The fraction of cells within the eight foci staining for CD3 (T lymphocytes) ranged from 34 to 57%, whereas the fraction staining for CD45/LCA (total leukocytes) ranged from 45 to 74%. The proportion of maternal cells within each focus was significantly correlated with the number of CD3-positive T lymphocytes but not with the number of CD3-negative leukocytes. We conclude that maternal cells, probably CD3-positive T cells, cross the maternofetal barrier and participate in VUE. (Am J Pathol 1993, 143:473–479)

Chronic villitis is characterized by an accumulation of chronic inflammatory cells in the fetally derived stroma of placental villi. A small percentage of cases have a recognizable infectious etiology, usually one of the TORCH group of organisms,[1] but the great majority of cases are idiopathic. These latter cases have been termed villitis of unknown etiology (VUE). VUE is an important cause of intrauterine growth retardation and recurrent reproductive failure and is one of the lesions associated with an elevation of midtrimester serum α fetoprotein.[2–6] Clinical sequelae of VUE generally parallel the extent of disease.

There are two outstanding questions regarding the pathogenesis of VUE. The first is the nature of the eliciting antigen. Whereas unrecognized infections can never be totally excluded, some have suggested an alternative hypothesis, that VUE might represent a type of host-versus-graft or graft-versus-host reaction.[5,7] Central to addressing the identity of the eliciting antigen is an understanding of the composition of the inflammatory infiltrate. Whereas a number of studies have provided phenotypic characterization of this infiltrate,[7–9] the second and most fundamental question regarding VUE is whether the villous inflammatory cells are derived from the fetus, the mother, or both. If they are exclusively fetal in origin, then reaction to exogenous antigen is the most probable pathogenetic mechanism. If they are at least partially maternal in origin, a more interesting scenario emerges where maternal cells cross the maternofetal barrier and respond to either exogenous microbial products or endogenous fetal antigens.

In this study, we have used two techniques, *in situ* hybridization with X and Y chromosome-specific probes and immunostaining for inflammatory cell markers to study the lineage of the villous stromal infiltrate in VUE and discover if a substantial proportion of these cells are CD3-positive maternal T lymphocytes.

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Materials and Methods

Cases of presumed VUE involving a male fetus were obtained from our recent files. The clinical charts of both mother and fetus were reviewed. In all cases, thorough serological studies excluded infection by organisms of the TORCH group. No infants showed clinical signs of congenital infection. Close inspection of multiple placental sections revealed no evidence of viral inclusions, toxoplasma cysts, or typical features seen with TORCH infection (i.e., villous plasma cells, stromal calcification, chronic membranitis, and funisitis). Finally, in selected cases (diffuse villitis), Warthin-Starry stains were performed to exclude spirochetes and bacteria. On this basis, the cases selected were classified as VUE. Severity of villitis was graded according to published criteria.[10] A total of six male plus two control female cases were studied. Paraffin blocks were serially sectioned at 4 μ and applied to silanized slides. Consecutive sections were stained as described below for the number of X chromosomes, CD3 antigen, the number of Y chromosomes, and CD45/leukocyte common antigen (LCA). The serial section approach was chosen after attempts at double staining using *in situ* hybridization plus immunoperoxidase were unsuccessful. Sections flanking these four slides were in some cases stained for other inflammatory cell markers. Slides from four male placentas showed adequate staining of X and Y chromosomes, CD3, and CD45/LCA. Suboptimal tissue morphology precluded further evaluation in one case. One of the two female placentas showed adequate morphology and staining for all markers. Details of the five cases evaluated are shown in Table 1.

Chromosomal *in situ* hybridization was performed using the Oncor-Light System (Oncor Inc., Gaithersburg, MD). Biotin-labeled probes against the DXZ1 α satellite locus on the X chromosome and both the DYZ1 α satellite and the DYZ3 classical satellite loci on the Y chromosome were used, the latter two as a probe cocktail. Briefly, sections on silanized slides were baked at 65 C overnight, incubated for 15 minutes at 37 C with a protein digesting solution, then washed, and dehydrated through serial alcohols and acetone. Slides were hybridized overnight at 37 C with biotin-labeled probe at 2 ng/ul of hybridization solution following rapid denaturation of probe and target DNA at 90 C for 10 minutes. Following hybridization, the slides were washed three times at low stringency (2× SSC 50% formamide) and once at high stringency (0.1× SSC), all at 37 C. The detection system utilized consecutive incubations with a blocking reagent, avidin, peroxidase-labeled anti-avidin, and diaminobenzidine. The capacity of the technique to differentiate between male and female cells was established using formalin-fixed, paraffin-embedded male and female tonsillar tissue.

Immunoperoxidase staining was performed using standard methods. Briefly, sections were deparaffinized to 70% alcohol and endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Sections were preincubated in 20% serum of the species from which the secondary antibody was raised and the primary antibody was applied. After overnight incubation at room temperature, the secondary biotinylated antibody was applied for 30 minutes. Staining was visualized using the Vector Elite System (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen. Sections were counterstained in dilute hematoxylin. The primary antibodies used were as follows. Leukocyte common antigen (CD45/LCA), mouse monoclonal antibody (Dako, Carpinteria, CA); T lymphocyte CD3 complex (CD3), polyclonal rabbit antiserum (Dako); monocyte-macrophage antigen (CD68), mouse monoclonal antibody (Dako); and macrophage-specific HAM 56, mouse monoclonal antibody (Enzo Diagnostics, Syosset, NY).

Foci of VUE were defined for the purposes of this study as discrete regions within each placenta containing 15 or more chronically inflamed nonstem villi within an area less than or equal to 10 mm in diameter. Two foci with VUE plus an additional control focus without VUE were aligned and marked on each

Table 1. *Clinical and Pathological Characteristics of VUE Cases*

Case no.	Infant sex	Gestational age	Grade of villitis*	Clinical history
1	Male	40	Severe	IUGR,† Gestational diabetes
2	Male	41	Severe	Infant with meconium peritonitis
3	Male	36	Mild	Premature rupture of membranes
4	Male	42	Moderate	Postdates
5	Female	38	Moderate	Premature rupture of membranes

* Criteria of Knox and Fox (ref. 10).
† IUGR: intrauterine growth retardation.

of the four serial sections (stained consecutively for X chromosome, CD3, Y chromosome, and CD45/LCA). Subsequent evaluation was performed manually using a 100-mm^2 eyepiece reticle at 400× total magnification. Five nonstem villi of less than 100 mm^2 total surface area and containing at least 100 evaluable cells were scored within each focus. Only nonstem villi that retained a circumferential lining of syncytiotrophoblast and lacked stromal necrosis were selected. For control foci, where individual villi did not contain 100 cells, five separate groups of contiguous villi totaling at least 100 stromal cells were evaluated. All villous stromal cells were scored for the presence of 0 to 2 positive X or Y chromosomal signals and the presence or absence of cell-surface staining for CD45/LCA and CD3. Cells at the margins of the villi, mostly trophoblast, were not counted. Results were expressed as the mean and standard deviation of the five individual (or groups of contiguous) villi enumerated for each VUE or control focus.

The data was further analyzed as follows. The percentages of XX- and Y-positive cells were corrected for plane of section by dividing the mean for each focus by the percentage of positively staining cells in female and male tonsils respectively. The percentage of maternal cells was then calculated by normalizing the percentages of XX- and Y-positive cells to 100%. In most cases, these totals were already close to 100% (range 86 to 119%). CD3-negative leukocytes were estimated by subtracting the percentage of CD3-positive cells from the percentage of CD45/LCA-positive cells for each focus. Pearson correlation coefficients were calculated between percent maternal cells and percent CD3-positive cells and between percent maternal cells and percent CD3-negative cells. Significance was evaluated using two-tailed t-tables.

Results

We first established that X and Y chromosome-specific probes could differentiate between male and female cells in paraffin-embedded sections. Because of the 4-μ plane of section (and other factors), not all female cells stain for two copies of the X chromosome and not all male cells stain for one X and one Y chromosome. Also, cells undergoing mitosis can have more than the expected number of X and Y chromosomes. It was therefore necessary to empirically determine the staining patterns of male versus female cells (Table 2). This was done using three types of controls. Because we believed that most cells in VUE were lymphocytes, we initially evaluated male and female tonsils. The results of tonsillar staining showed that 74% of male cells stained for one Y chromosome, whereas 61% of female cells stained for two X chromosomes. Next, we tested the VUE placenta from a female infant (case no. 5), where both fetal and maternal cells would be female. Results from VUE and control foci in this

Table 2. *Percentage of Villous Stromal Cells Staining Positively for X and Y Chromosomes, CD3, and CD45/LCA*

Case no.	Sex	Focus	X-Chromosomal probe			Y-Chromosomal probe		CD3	CD45/LCA
			XX*	XO	OO	OY	OO		
1	Male	1	30.4 ± 10.41†	24.6 ± 5.23	30.2 ± 11.12	27.4 ± 8.02	66.0 ± 8.40	56.8 ± 4.27	74.2 ± 0.84
		2	30.0 ± 3.61	38.6 ± 6.19	19.4 ± 5.46	35.4 ± 5.55	61.0 ± 4.85	40.8 ± 11.10	59.2 ± 4.27
		Control	0.9 ± 1.04	25.3 ± 1.71	73.8 ± 2.75	68.3 ± 2.63	27.8 ± 3.00	2.0 ± 3.03	14.8 ± 2.95
2	Male	1	33.2 ± 6.14	34.6 ± 7.44	25.6 ± 8.91	40.2 ± 4.21	58.4 ± 5.41	33.8 ± 9.15	44.8 ± 6.34
		2	29.6 ± 5.23	47.0 ± 5.52	15.3 ± 8.97	44.0 ± 4.30	53.6 ± 4.72	33.8 ± 8.67	50.6 ± 5.13
		Control	3.1 ± 1.58	46.0 ± 7.36	50.4 ± 6.84	64.8 ± 5.26	35.0 ± 5.57	3.2 ± 1.81	18.2 ± 3.12
3	Male	1	54.0 ± 4.55	32.8 ± 6.30	2.5 ± 2.08	22.0 ± 8.42	74.8 ± 12.26	56.8 ± 3.70	67.6 ± 4.34
		2	41.4 ± 14.00	34.0 ± 8.46	13.9 ± 9.30	31.8 ± 23.82	68.2 ± 23.80	51.0 ± 13.40	67.4 ± 7.27
		Control	3.3 ± 0.91	67.8 ± 4.21	29.0 ± 5.00	69.0 ± 5.34	29.8 ± 3.42	1.9 ± 0.76	20.4 ± 2.30
4	Male	1	37.4 ± 4.72	54.2 ± 8.50	6.6 ± 5.16	42.6 ± 9.86	53.2 ± 9.09	38.8 ± 6.38	63.4 ± 8.39
		2	34.0 ± 3.81	55.0 ± 4.30	11.2 ± 2.17	48.2 ± 5.31	50.8 ± 6.06	40.6 ± 5.55	60.8 ± 4.55
		Control	3.1 ± 2.35	77.0 ± 7.75	18.6 ± 7.64	76.4 ± 2.61	22.6 ± 3.36	0.4 ± 0.89	15.8 ± 7.53
5	Female	1	65.4 ± 4.77	29.4 ± 5.03	3.7 ± 3.78	0	100	60.2 ± 6.42	65.4 ± 3.98
		2	58.8 ± 3.63	35.0 ± 3.94	5.3 ± 6.46	0	100	48.8 ± 8.50	62.0 ± 6.04
		Control	59.8 ± 5.45	31.6 ± 1.52	5.9 ± 2.57	0	100	2.0 ± 0.92	9.6 ± 2.19
Female tonsil					61.3 ± 5.91	27.8 ± 3.30	9.3 ± 3.10	0	100
Male tonsil					5.4 ± 1.09	75.0 ± 6.73	15.6 ± 5.81	73.8 ± 6.08	25.0 ± 6.78

* XX = 2 copies of X; XO = 1 copy of X; OO = no chromosomal staining; YO = 1 copy of Y.
† Mean and standard deviation for five villi; 100 cells counted for each.

placenta showed that 59 to 65% of cells expressed two copies of the X chromosome, in close agreement with the results in tonsils. Finally, we evaluated the four non-VUE control foci in male placentas with VUE. A single copy of the Y chromosome was detected in 65 to 76% of cells from these cases, again showing close agreement with tonsillar controls. The final possible control, evaluating areas of villitis in male placentas that lack female cells, could not be done because, as shown below, all foci of villitis contained maternal cells.

Next, we evaluated the X and Y chromosome profiles of cells in male cases of VUE. The results obtained from analysis of 40 affected villi in eight separate foci from four different cases ranging from mild to severe in severity were surprisingly uniform. Table 2 shows means and standard deviations within each focus for each chromosomal staining profile. Between 30 and 54% of cells in foci of VUE contained two copies of the X chromosome. Standard deviations for the proportion of XX cells were generally low (six of eight less than 7.0, maximum 14.0), suggesting uniform involvement of villi within each focus. Accepting that between 0.9 and 5.4% of male cells will have two X chromosomes (presumably due to mitosis) and comparing these numbers to the 59 to 65% positive rate in exclusively female tissues, this indicates that a major fraction of the cells in foci of VUE are maternal in origin and must have crossed the maternofetal trophoblastic barrier. On the other hand, 22 to 48% of cells in VUE foci contained a Y chromosome and hence were fetal in origin. This latter result can be compared to the finding of 65 to 83% of Y chromosome-positive cells in non-VUE foci. An example of the observed pattern of X and Y chromosomal staining in VUE is shown for serial sections of the same villus in Figure 1, A and C.

We then studied the composition of the inflammatory infiltrate by staining for CD45/LCA (total leukocytes) and CD3 (T lymphocytes). Other investigators[8,9] and our own unpublished results suggest that B lymphocytes and plasma cells are generally not present in foci of VUE. We found that the difference between the percentage of CD45/LCA-positive and CD3-positive cells was almost entirely accounted for by cells that stained with both CD68 and HAM 56 and hence were of presumed monocyte-macrophage origin (results not shown). Table 2 shows that CD3-positive T lymphocytes were rare in noninvolved villi (1 to 3%) but prevalent in foci of VUE (34 to 57%). Figure 1B shows frequent CD3-positive cells in a serial section from the same affected villus, whose X and Y chromosomal staining pattern is illustrated in Figure 1, A and C.

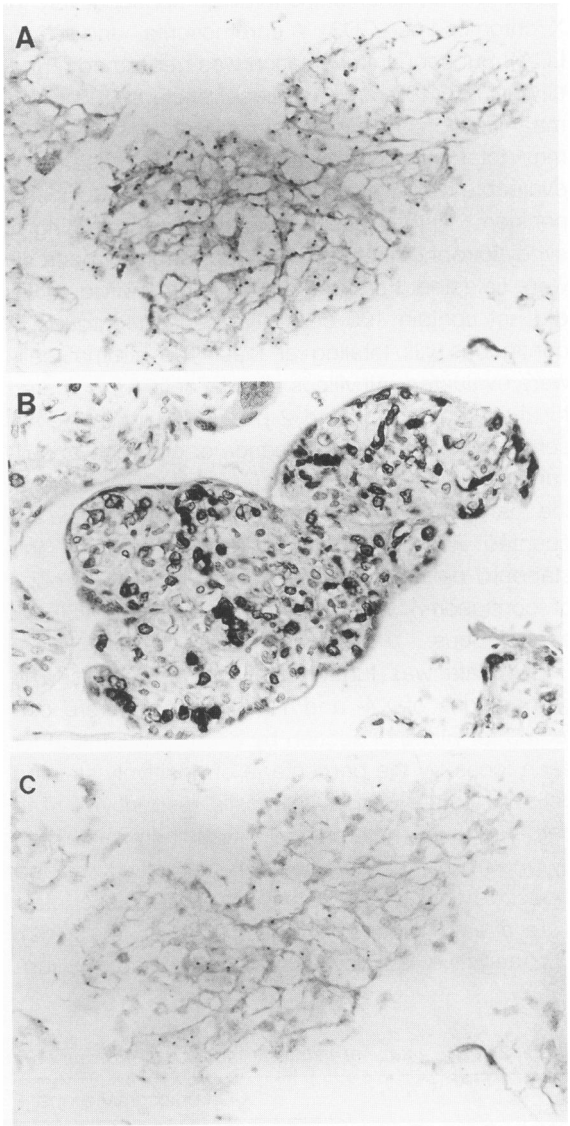

Figure 1. *X and Y chromosome-specific in situ hybridization and CD3 staining in VUE. The three photographs are from serial sections of a single villus from case no. 3.* **A.** *X chromosome in situ hybridization: multiple cells throughout the villus show two dark dots overlying the nucleus, marking them as being of maternal origin.* **B.** *CD3 immunostaining: a substantial proportion of infiltrating cells are darkly stained CD3-positive T lymphocytes.* **C.** *Y chromosome in situ hybridization: only a minority of cells show a single dark dot overlying the nucleus, marking them as being of fetal origin.*

In the absence of double staining, it is not possible to state definitively whether the marked increase of T lymphocytes in foci of VUE is accounted for by maternal cells, fetal cells, or both. To gain some insight into this question, we looked more carefully at the composition of the inflammatory infiltrate. The average proportions of XX-bearing maternal and Y-bearing fetal cells in each of the eight foci of VUE were corrected for plane of section and normalized

to 100% as described in the Materials and Methods section. This calculation revealed that between 50 and 75% of cells within villi with VUE are of maternal origin. The calculated proportion of maternal cells was then plotted against the percentage of CD3-positive cells (Figure 2A) and the percentage of non-CD3-positive leukocytes (% CD45/LCA-positive minus % CD3-positive, Figure 2B) for each of the eight foci of VUE. Correlation between the percentages of maternal cells and leukocyte subsets was determined by the Pearson rank correlation test. A significant positive correlation was found between the percentage of maternal cells and the percentage of CD3-positive T lymphocytes ($r = 0.74$, $P < 0.05$), whereas there was a nonsignificant inverse relationship between maternal cells and non-CD3-bearing leukocytes ($r = -0.34$, NS).

Discussion

Isolated foci of VUE are found in 8 to 14% of all placentas.[2,10] Much less commonly, when extensive, VUE can cause significant pathology such as intrauterine growth retardation and recurrent reproductive failure.[3,5] Two competing schools of thought have dominated consideration of VUE (discussed in ref. 11). First are those who stress the obvious parallels between VUE and the villous inflammation associated with TORCH-type infections. These observers argue, by analogy, that VUE is most likely to be a fetal response to microbial antigen.[1,12] It is notable that despite intense scrutiny over a number of years, no evidence implicating any infectious agent has been found for this quite common lesion. The second group of investigators have suggested that VUE represents an allograft reaction between maternal and fetal cells. Supporting this hypothesis are the tendency of VUE to recur in some patients,[3,5] an association with markers of maternal autoimmunity,[5] the predominance of CD4-positive T helper cells and Ia antigen-bearing macrophages,[7] and by default the absence of any evidence for infection. It is often assumed that the first hypothesis implies that VUE is an exclusively fetal immune response, whereas the second hypothesis indicates that only maternal cells play a role. It is important to stress that there is no a priori reason for this bias. An infection might elicit a primarily maternal response, whereas an allograft reaction could be dominated by the fetal response to a few maternal cells that may have wandered across the maternofetal barrier. The important questions are: do maternal cells enter fetal tissues during VUE and, if so, what are the long-term consequences of this intermingling of histoincompatible immune cells?

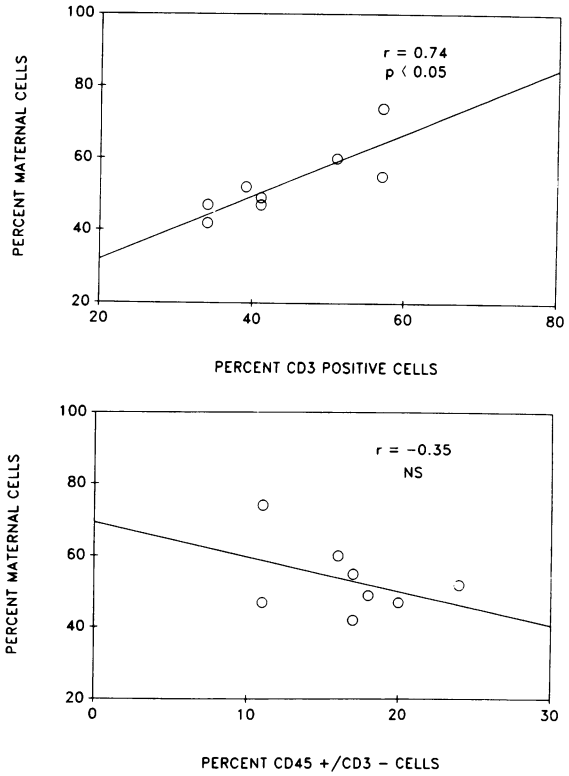

Figure 2. *Correlation between the proportion of maternally derived cells and CD3 antigen status in foci of VUE. The eight data points on each graph represent means derived from the five villi evaluated for each of the eight independent foci of VUE from cases 1 to 4. The graphs plot the percentage of maternal cells (calculated as described in the text) versus the percentage of total villous stromal cells staining positively for CD3* (**A**) *and the percentage of total villous stromal cells staining positively for CD45/LCA, but lacking CD3 (%CD45 to %CD3).*

The study of VUE has been hampered by the inability to make the diagnosis prospectively. For this reason, fresh tissue is rarely available for study. Recent advances have made it possible to determine directly the percentages of maternal and fetal cells in VUE using X- and Y-specific chromosomal probes on archival paraffin blocks. This technique, chromosome-specific *in situ* hybridization, has primarily been utilized for the analysis of karyotypes in malignant tumors and spontaneous abortions (reviewed in ref. 13). An earlier application of this technique, however, was for the analysis of experimental chimeras.[14] It is in this context that chromosomal *in situ* hybridization was the natural approach for addressing the long-standing question of the origin of the inflammatory infiltrate in VUE. The results of this study conclusively demonstrate for the first time that maternal cells are a major component of the inflammatory infiltrate in VUE.

Having established that maternal cells participate in VUE, we sought to characterize further the nature

of the infiltrating cells. CD3 is a component of the T cell antigen receptor and, hence, is a specific marker for antigen-specific T lymphocytes.[15] The marked increase and predominance of CD3-positive cells in foci of VUE plus the strong positive correlation between the numbers of maternal and CD3-positive cells strongly suggest that VUE is the consequence of invading maternal T lymphocytes. Consistent with this interpretation, our immunostained sections showed CD3-positive cells surrounding and crossing the trophoblastic barrier (data not shown). Rare T lymphocytes were also seen in fetal vessels at foci of VUE. We have not stressed these immunohistological observations because, in the final analysis, there is no way to prove using immunostaining alone whether the flow of cells is from maternal to fetal tissue or vice versa. Likewise, cells in the fetal circulation could be either fetal T lymphocytes traveling to the villus or maternal T lymphocytes moving toward the fetus. Unfortunately, determining the lineage of cells within fetal vessels was beyond the resolution of the *in situ* hybridization method.

Whether T lymphocytes in the fetal circulation are fetal or maternal may vary in individual cases and could have profound implications for pregnancy outcome. Invasion of the fetus by maternal cells is a well-known phenomena in rodent pregnancies and leads to a form of lethal graft-versus-host disease known as the runting syndrome.[16] In cases of severe growth retardation, stillbirth, or recurrent fetal loss associated with VUE, maternal cells could cause fetal pathology by this mechanism.[17] On the other hand, a prompt and aggressive fetal immune response to invading maternal cells might serve to limit the spread of maternal cells to the fetus. A second mechanism that might limit access of maternal cells to the fetal circulation is the striking obliteration of fetal vessels that commonly occurs in cases of VUE and that serves as a useful histological clue for identifying foci of VUE at low power in tissue sections.

Questions that cannot be directly answered by this study are the nature of the eliciting antigen and the pathogenesis of the lesion. As stated above, the finding of maternal T lymphocytes in VUE does not exclude an infectious etiology. In fact, preliminary results from our laboratory suggest that maternal T lymphocytes are also found in a small number of the most severely affected villi of placentas with congenital syphilis and cytomegalovirus infection. Regarding the pathogenesis of the lesion, we would suggest that the pathological lesion, VUE, must represent multiple independent foci of maternal cell infiltration because it typically involves multiple contiguous groups of villi, not all of which belong to the same villous tree. This would imply that whole groups of villi become susceptible to infiltration by maternal leukocytes, perhaps due to local conditions that alter the trophoblastic barrier. Possible mechanisms might include altered expression of adhesion molecules by either leukocytes or trophoblast, regional ischemic damage to trophoblast, or utilization of perivillous fibrin, which commonly accompanies VUE, as a bridge for invading leukocytes. Each may be operative in individual cases. We propose the following working model. Initially, maternal cells enter individual villi where they encounter foreign transplantation antigens. The early maternal immune response leads to either the closure of fetal vessels or the recruitment of fetal lymphocytes that attack maternal cells. In some cases, the local immune response gains sufficient momentum, perhaps through the elaboration of cytokines, to promote the infiltration of surrounding villi leading to the lesion we recognize as VUE. In selected cases, the immune stimulation is sufficiently intense to cause severe villitis with profound consequences for the fetus. Factors influencing the development of severe villitis could include the nature of the eliciting antigen or the frequency of circulating antigen-specific maternal T cells. One reason for a high frequency of specific maternal T cells might be prior exposure, and this could account for recurrent villitis. Another important consideration is that, as mentioned above, a weak or inadequate local immune response might allow unimpeded spread of maternal cells resulting in fetal engraftment and graft-versus-host disease. This possibility is being evaluated in ongoing studies in our laboratory.

In conclusion, we have demonstrated that maternal cells are a major component of VUE and that many of these infiltrating cells are T lymphocytes. This, together with other observations showing a predominance of the CD4 helper/inducer T lymphocyte subset and diffuse local Ia antigen induction, reveal VUE to be an interesting example of maternal-fetal cell interaction that has potentially significant implications for pregnancy. Future work needs to specifically address the precise nature of the eliciting antigen and the specific factors that predispose some women to more severe lesions.

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