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## Discussion

DR. ARNOLD G. DIETHELM (Birmingham, Alabama): Wayne, I enjoyed your paper a great deal and it's an interesting and yet very complex set of experiments that you have presented to us. Let me limit my comments to maybe some of the mechanistic aspects. Do you think the cell needs to leave the thymus in order to achieve this level of tolerance? In other words, you're injecting a cell into the thymus, does that cell undergo a change and then leave the thymus or does something happen in the thymus that other cells that leave the thymus are now tolerogenic. And where do these cells go if they do leave the thymus? It would seem to me that if there's clonal deletion, it couldn't all happen in that very short period of time. Possibly I'm wrong. And what is the timing of the ALS and the tolerogenic effect of thymus? You mentioned, I believe, 21 days. Is that an important time event or can it be sooner or later? Obviously the time between the injection of the ALS and the tolerogenic result is critical when one considers any aspect of clonal transplantation. I very much enjoyed your paper. I think you're on to a very complex subject. It's going to be interesting to see how all of this plays out and whether or not the cells in the thymus have to leave the thymus or if something else happens to make the animal tolerogenic.

DR. JAY C. FISH (Galveston, Texas): The Australian, Kevin Lafferty, demonstrated 15 years ago that in a murine model if you take the thyroid and parathyroid out and culture it for 28 days in high oxygen that all the passenger leukocytes die off and you're left with a pure culture of follicular cells that bear only Class I antigens. Those cells can be transplanted in the mouse successfully without immunosuppression. Unfortunately neither he nor anyone else has been able to duplicate this finding in higher order species. In addition it has been difficult with other tissues. These studies demonstrate the importance of Class II antigens in donor tissue to stimulate the allergenic response. What we've seen today is the importance of Class II antigen cells in the donor tissue to produce tolerance. A certain inner logic of that might be predicted. What would be interesting to know is if it works as well with other tissues such as skin and how it works in higher order animals. I, too, am interested in knowing the fate or the final resting place of the cells in the inoculum to the thymus.

DR. FRANCIS T. THOMAS (Greenville, North Carolina): Dr. Flye's work is really a very important extension of previous studies in which he has defined parameters of tolerance induction. Adult tolerance, the Holy Grail of transplantation, has been expanded strikingly by studies such as these in the last few years. At the recent International Transplant Congress a special session on tolerance was held. The summary report of the session cited tolerance as perhaps the most important area of transplant research today. Our group have induced long-term tolerance in incompatible kidney grafts in Rhesus monkeys with donor antigen. Tolerance is defined as long survival of incompatible kidney grafts up to 2–3 years without any chronic immunosuppression. The animals generally survive in extraordinary state of health unlike that of animals with chronic suppressive drugs. Long-term serial immune studies have now been done up to 3 years in these animals and reveal some interesting findings some of which are not unlike some of the findings seen today, but some are different. This is probably to be expected in that there is perhaps more than one form of adult tolerance seen. Monkeys similar to humans have an atrophied thymus which cannot clearly be identified grossly at autopsy and thus this may not be central to the tolerance mechanism as it is in the rat model which Dr. Flye has described. Wayne, do you think that the differences in the tolerance model mechanism we see here relates to the species studied or the state of the thymus function in these two species. We've always felt that our adult tolerance model generated extrathymic tolerance and perhaps the tolerance developed locally in the graft. Have you seen any evidence for this in your model? Secondly, you may recall, we reported on a deletion of both the cytotoxic T-cell activity as well as CTL precursors four years ago findings strikingly similar to what you reported today, and we felt this tolerance was therefore a deletional one. However, serial immune studies done subsequently on several primates up to 2–3 years post-transplant with well-functioning grafts have shown a gradual return of CTL precursors without any evidence of rejection. Therefore, this is a dynamic process and I think one we don't understand and this led us to perhaps the most important and central issue here, that of the potential for chimerism or the existence of chimerism in these studies. In addition, the conclusions of these studies are so important that I think we need to be sure we're not dealing with any artifacts. Are you sure of the purity of these cell preparation which, I believe, you've commented on already? This is a particularly difficult problem that we've encountered in the lab in separating these preparations into pure T-cell and non-T-cell preparations. We found, for example, that the DR portion that Dr. Flye has infused inducing tolerance has both a tolerogenicity component and also an antitolerogenic component. Finally, although not discussed here but discussed in the paper, I would guess your model does not finally rule out the so-called veto cell type mechanism which we have postulated to explain this striking tolerance. Veto activity by non-T cells has been reported and our own group have found the principal tolerogenic cell in the veto assay to be a CD3 negative and thus not a classic T lymphocyte as reported by Rick Miller's group. Our results tend to agree with Tom Starzl's recent reports from long surviving human transplant recipients in that the principal tolerogenic cell is probably, in our opinion, a donor dendritic cell with a CD2

positive, CD8 positive, CD16 positive phenotype whose identity has been confused with DR cells by the presence of levels of dim staining on FACS analysis. I wonder if you could comment on this subject. The subject of demonstration of chimerism as mentioned is one which we're all moving towards and seems to be clearly one which can be achieved whether we're talking about the liver transplants of Dr. McDonald or some of the work which Dr. Diethelm has done in the human with the donor bone marrow, or our work in the higher primates with this.

DR. KRON: Dr. Flye, would you please close and on your way up Dr. McDonald has a key question for you.

DR. McDONALD: Dr. Flye, on your way up I'd just like to ask if the intrathymic injection induction of tolerance has been shown in any species other than the rat.

DR. WAYNE FLYE (Closing Discussion): Let me answer Dr. McDonald's question first. There are no reported studies that intrathymic tolerance has been effectively carried out in other than a rodent model. It has been suggested that attempts have been made in the dog by another group and tolerance was not achieved. Dr. Thomas alluded to one difference between the rodent model and higher animals particularly when you get to the level of monkey and man. With adolescence, involution of the thymus occurs and that possibly new extrathymic pathways are probably utilized for thymocyte maturation. Could the thymic environment be altered to prevent involution? There are some exciting reports using various hormones to increase the cellular proliferation within the thymus. Theoretically, it is possible that stimulation of the involuted thymus could then allow tolerance induction. The thymic stroma, including dendritic cells, remains after thymic atrophy of the thymus. Therefore, if lymphocyte trafficking redeveloped, thymocyte maturation could potentially occur. An important question is whether we are examining a tolerance phenomenon different from that reported before with blood transfusions or with bone marrow as Dr. Thomas and Dr. Diethelm have alluded to. Microchimerism or persistence of the donor cells appears to be important for development of tolerance with both these conditions. We have achieved graft tolerance by giving cells treated with UVB irradiation into the thymus. We demonstrated that these cells do not survive more than 24 hours in culture. Thus, they appear not able to survive *in vivo* and, therefore, would not establish microchimerism. In addition, cells injected into the spleen, the portal vein and subcutaneously do not replicate this effect. It again points to the fact that alloantigen is needed in the intrathymic environment. What happens to the cells when they're injected into the thymus? We have not been able to demonstrate Lewis splenocytes after about a week in the thymus. However, intrathymic islet transplants function and persist for a long time within the thymus. When thymectomy is performed three days after intrathymic injection of splenocytes, subsequent cardiac grafts are rejected in normal time while thymectomy after seven days allows indefinite graft acceptance in the majority of recipients. However, we cannot be absolutely sure that some splenocytes have not migrated out of the thymus before thymectomy. Our studies indicate that CTL clonal

energy or elimination is an important result of the intrathymic alloantigen exposure. In a different experimental model, Matzinger showed that antigen presented in the context of professional antigen-presenting cells, that is, the dendritic cells and the macrophages that I've been referring to here, you get intrathymic tolerance. If other cells, such as B cells, present antigen, you don't get tolerance. Alloantigen as peptide shed from the cells or as cells phagocytosed and antigen reexpressed on the antigen-presenting cell surface reproduces conditions similar to that for self antigen in the thymus for selection of thymocytes. Dr. Fish mentioned Dr. Lafferty's important work. Graft acceptance appears to result from a different mechanism. Two signals are thought to be necessary. In addition to antigen presentation, molecules such as interleukin-2 act as the second signal that triggers the responding cells. If you culture the cells or the organ, such as thyroid, for a period of time you eliminate

dendritic cells and inhibit an immune response. However, when class II cells are placed in the thymus, they interact with immature thymocytes that are susceptible to inactivation or elimination in contrast to mature lymphocytes in the periphery that would be stimulated. Dr. Thomas, we're quite familiar with your veto cell work. We know that the cells that we inject in the thymus are greater than 95% Class II<sup>+</sup> cells. Veto cells would have to persist in the periphery to induce tolerance. Our *in vitro* assays do not show suppression of MLC responses by long-surviving graft recipient cells that would indicate the presence of veto (donor cells) or suppressor (recipient) cells. The Pittsburgh group has found evidence of persisting donor dendritic cells. However, the question is, which is the cart and which is the horse? Do these cells migrate out of the organ and persist in the recipient because of suppression or do they induce unresponsiveness? It is still not clear which comes first.