

Immunotherapy of the IR162 Rat Myeloma

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The IR162 transplantable rat myeloma is a spontaneous tumor in a Louvain rat (2, 3). The tumor has been established in cell culture as the Ta4 line, and tumor cells grown *in vitro* and *in vivo* have the morphology of plasmablasts (8, 10, 11). The tumor cells secrete immunoglobulin E *in vitro* and *in vivo*, and express IgE on their cell surfaces (9, 10). The growth and metastasis of the tumor has been well described, and the tumor cells have been extensively examined *in vitro* for their sensitivity to a number of cytotoxic and immunomodulatory agents (8-11). The IR162 tumor was therefore considered a potential model for immunotherapy of multiple myeloma.

Nonspecific immunity has often been shown to be due to natural killer cell activity (6, 14, 18), so nonspecific immunity was examined by the intravenous injection of normal spleen cells into tumor bearing animals, and the induction of lymphokines, which have been reported to enhance natural killer cell activity (18, 20). Specific immunity was evaluated by the use of spleen cells from tumor bearing animals and immunization with live and killed tumor cells.

Materials and Methods

Single injections of normal spleen cells. Young adult Lou/M rats 3 to 4 months of age were injected subcutaneously on the dorsum with 3 million IR162 tumor cells from a disaggregated subcutaneous tumor suspended in 0.5 ml Eagles Minimum Essential Medium (MEM). On day 8 after injection, when no tumors were yet palpable, 5×10^7 nucleated spleen cells from normal rats of the same sex and age were suspended in MEM and injected intravenously via the tail vein. Control animals received intravenous diluent alone. Animals were observed twice weekly for the appearance of tumors. Tumor size was measured by calipers and expressed as the product of two tumor diameters.

Multiple injections of normal spleen cells. Eighteen 3 to 4 month old Lou/M rats were injected with tumor cells as above and divided into 3 groups. Group I consisted of the control animals, which were intravenously injected with MEM on day 8. Group II received 5×10^7 spleen cells from untreated rats on day 8. Group III received spleen cells from untreated rats on days 1, 8, 15, and 22. Tumor size, expressed as the product of two tumor diameters, was observed at days 28 and 35; and tumor weight was determined at autopsy on day 40. Groups were compared by Student's T test.

Spleen cells from tumor-bearing rats. The protocol was the same as for the single normal spleen cell injections except that Group I (9 rats) received 5×10^7 spleen cells from rats which had been subcutaneously inoculated with the tumor on day 0. Group II animals (4 rats) were controls and received an intravenous injection of diluent on day 8. Tumor weights at autopsy were compared using Student's T test.

Lymphokine induction followed the procedure of Ruscetti *et al* (17). Five groups of 5 young adult male Lou/M rats were treated as follows. On day -7 groups A, C, and D received an 0.5 ml tail vein injection of an oil in water emulsion containing 2 mg killed acetone dried *Mycobacterium tuberculosis* kindly donated by Dr. Ruth Neta, University of Notre Dame. Group B received the same vaccine

intraperitoneally, and Group E was untreated. On day 0 all rats were inoculated subcutaneously with 1 million IR162 tumor cells in 0.5 ml MEM. Groups A and B received single 0.5 ml intravenous challenge injections containing 100 mg old tuberculin (Jensen) on day 8. Group D received 5 mg old tuberculin(OT) intravenously on days 1, 4, 8, 11, 15, and 18. Groups C and E were unchallenged. Tumor development was observed as above.

Two approaches were taken to evaluating the development of specific immunity to the IR162 myeloma. First, 3 to 4 month old Lou/M rats were injected subcutaneously with 1 million Ta4 cultured IR162 tumor cells, which had been killed by 20 minute exposure to Mitomycin C (20 μ g/ml) and emulsified 1:1 in Freund's Complete Adjuvant. Cultured tumor cells were chosen because they would not contain other cell types found in tumor masses. Rats subsequently received 3 biweekly injections of Ta4 cells alone. For allogeneic controls, Sprague-Dawley rats were immunized with 10 million live tumor cells twice, 3 weeks apart. Previous work (8) had shown that this dosage would not produce tumors in Sprague-Dawley rats, but the initial growth of tumor cells should provide increased stimulation of the immune system. In the second approach rats were given a subcutaneous injection of 5×10^5 tumor cells, which caused tumors in less than half the animals; and tumors were allowed to develop. Then the tumor free rats and another group of rats which had not previously received tumor cells were again given that inoculum and compared for the frequency of tumor development to see if the initial failure to develop tumors resulted in immunity.

Antibody mediated cell cytotoxicity was evaluated by the method of Ohanian (16). Ta4 cells were washed twice, suspended in veronal buffered saline (VBS), which had been supplemented with 0.5 mM $MgCl_2$ and 0.1% gelatin, and diluted to 10^5 /ml. A 1/10 dilution of serum (0.1 ml) from immunized or control rats and tumor cells (0.1 ml) were incubated together at 30C for 30 minutes to allow antibody to bind to tumor cells; then cells were washed twice in VBS to remove unbound antibody. A 1/10 dilution of rabbit complement (0.1 ml) was added to the cells and incubated at 37C for 30 minutes. Finally 0.1 ml 0.4% trypan blue dye was added and live and dead tumor cells were counted in a hemocytometer after 4 minutes. Data were reported as percent killed.

Cell mediated cytotoxicity was evaluated by preparing cultured Ta4 tumor cells as above, suspending them at 10^5 /ml in RPMI 1640 medium supplemented with 10% fetal calf serum, to which nucleated spleen cells were added at ratios of 2:1, 10:1, and 100:1, and incubated 5 hours at 37C, which is an appropriate time for NK cell killing (6). Spleen cells were disaggregated through a mesh screen, purified on Histopaque (Sigma Chemical), and washed twice in balanced salt solution. After incubation, trypan blue dye was added as above, and live and dead tumor cells were counted. Note that because of the difference in size, tumor cells may be easily differentiated from spleen cells. Data were expressed as percent killed and total remaining tumor cells.

Results

Intravenous injection of normal spleen cells increased the tumor free period and survival of Lou/M rats injected with IR162 tumor cells. In one experiment involving 10 control and 6 experimental animals, spleen cell therapy increased the mean survival from 55 to 74 days, excluding one treated animal which died without evidence of tumor at 280 days. In a second experiment control rats had a mean survival of 70 days, and 1 of 8 remained tumor free when the experiment was terminated at 180 days; experimental animals had a mean survival of 121

days with 4 or 8 tumor free at 180 days. The longer survival of controls in the latter experiment probably reflected a slightly lower viability of the initial tumor cell inoculum. Other experiments have shown that the state of tumor development, controlled by inoculum size and day of treatment, is critical to the success of spleen cell therapy.

Multiple spleen cell injections beginning on day 1 after tumor inoculation significantly ($p < .05$ by Student's T test) reduced tumor weight at autopsy (day 40) to 15 g compared to 18 g with one injection or 35 g for untreated animals. Significant reduction in tumor size was also observed at days 28 and 35 (Figure 1).

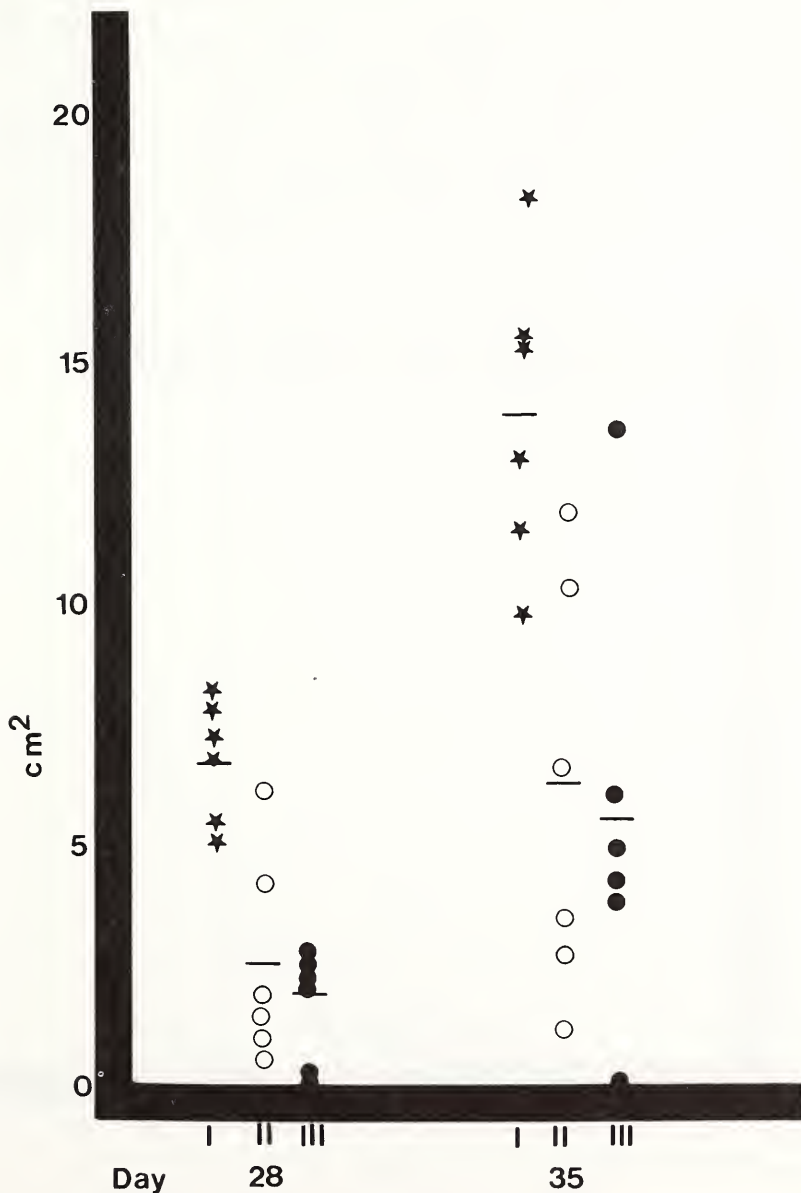


FIGURE 1. Effect of normal spleen cells on tumor development. (-) indicates the mean value. Tumor size is expressed as the product of two diameters. Group I was untreated, II received a single injection of spleen cells on day 8, III received multiple spleen cell injections. II and III differ significantly from group A ($p < .05$ by Student's T test).

Lymphokine induction by immunization to *Mycobacterium tuberculosis* and challenge with OT inhibited tumor development (Figure 2). Tumor weight at autopsy showed a similar pattern. The unchallenged groups C and E had mean tumor weights of 18.8 and 14.0 grams respectively. The challenged groups, A and B (100 mg OT intravenously) and D (repeated 5 mg challenges) had mean tumor weights of 10.9, 2.7, and 7.2 grams. Although differences between challenged and unchallenged groups were apparent, they were not significant at the 5 percent level using Student's T test.

Since nonspecific methods proved effective in inhibiting the tumor, and some spleen cell appeared to be responsible, spleen cells from tumor inoculated animals were evaluated as *in vivo* therapy (Figure 3). In contrast to the results with normal spleen cells, cells from tumor bearers significantly enhanced rather than inhibited the tumor growth ($p < .05$ by Student's T test).

When low tumor inoculum was used and tumor appearance in animals which had once failed to develop tumors was compared to animals which had never been exposed to the tumor, there was no apparent or significant difference in the fraction which developed tumors nor in the time for tumor development.

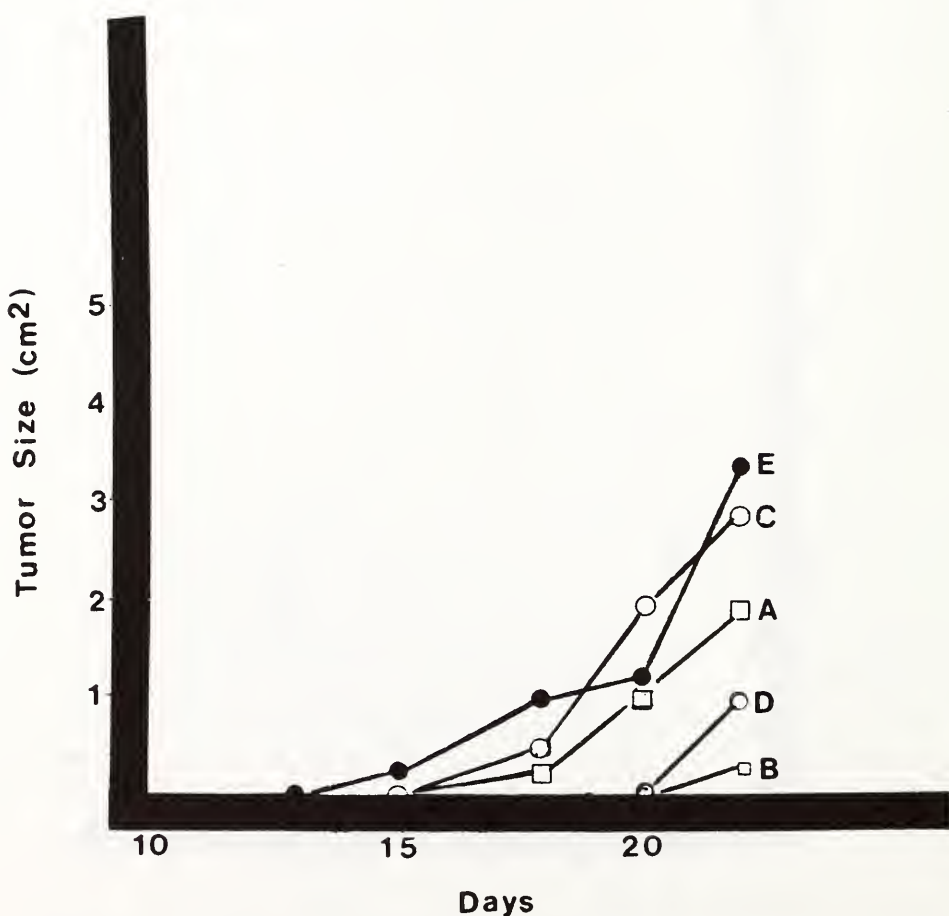


FIGURE 2. Effect of lymphokine induction on tumor development. Points represent mean tumor size expressed as the product of 2 diameters. Group A—i.v. immunization, i.v. challenge (100mg OT), Group B—i.p. immunization, i.v. challenge (100mg OT), Group C—i.v. immunization, no challenge, Group D—i.v. immunization, repeated low dose (5mg) challenge i.v., Group E—no treatment.

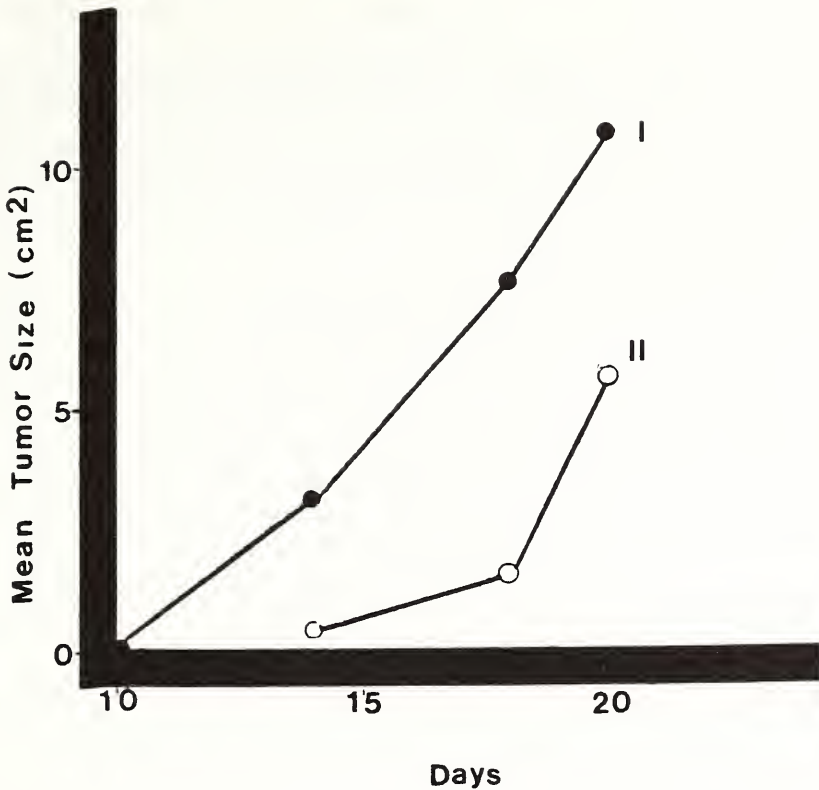


FIGURE 3. *Effect of spleen cells from tumor bearing rats. Group I received spleen cells from rats which were inoculated with a subcutaneous tumor 8 days before. Group II was untreated.*

Rats were immunized with killed tumor cells and evaluated for antibody that could kill tumor cells in the presence of complement. Rabbit anti-rat antibody and rat antibody against the major histocompatibility antigens of the Lou/M rat, anti-AgB2, prepared according to the procedure of Shinohara (19), were used as positive controls; normal rat serum was used as a negative control. Rabbit complement was found to give much better results than guinea pig complement with the rat antibody. Data were presented as percent of cells killed, as judged by trypan blue exclusion, and greater than 35% kill was considered positive. No control syngeneic (Lou/M) or allogeneic (Sprague-Dawley) rats produced cytotoxic serum. Two of 5 syngeneic immunized rats had cytotoxic antibody as did 3 or 8 allogeneic rats. When immunized Lou/M rats were killed and serum was tested at various times during the immunization procedure, 1 of 2 sera was positive at 1 week, 2 of 2 at 3 weeks, and 1 of 5 at 6 weeks. Therefore, the development of cytotoxic antibody occurred but was sporadic in immunized syngeneic and allogeneic rats. Sensitivity of the tumor cells to anti-AgB2 antiserum indicated that the tumor cells were susceptible to complement-mediated antibody killing, and that the cells expressed histocompatibility antigens.

Examination of spleen cells from immunized rats above indicated a slightly increased cell mediated cytotoxicity using 100:1 ratio of spleen to tumor cells over that observed with a 2:1 ratio, but there was no significantly increased cell mediated killing in immunized rats. A similar experiment in which rats were killed at two day intervals up to 2 weeks after tumor inoculation, also failed to show enhanced *in vitro* cell mediated killing by spleen cells.

Discussion

Multiple myeloma in humans is a widespread disease which may be treated with cytotoxic drugs but is seldom cured (7). Therefore, there has been interest in immunotherapy of the disease. Mouse plasmacytomas were responsive to specific immunotherapy (1,5,12), but the presence of virus in virtually all mouse cells and the response of the immune system to viruses makes the results somewhat more difficult to interpret.

The rat myeloma cells are virus free as far as is known (4), and the growth and metastasis of the transplantable IR162 myeloma has been thoroughly characterized (2,3,8,11). Therefore, we have attempted to evaluate the responsiveness of this model to nonspecific and specific immune modulation *in vivo*.

The tumor developing at a subcutaneous site was shown to be susceptible to inhibition by intravenously injected normal spleen cells, and multiple injections begun at day 1 were more effective than a single injection at day 8. Tumor size (expressed as the product of 2 diameters) has been shown to increase linearly with time once the tumors become palpable (11). Therefore, although the tumors were not palpable at day 8, it is reasonable to assume that the spleen cells travelled to the subcutaneous site and killed tumor cells, thus reducing the tumor burden. This implies the presence of a cell type in the normal spleen which can kill IR162 cells. The most likely candidate for that cell would be natural killer of NK cell, which is found at high levels in the spleens of rats from 2 to 18 months of age (6). Using our procedure for *in vitro* cell killing, which is admittedly not very sensitive, we were unable to find evidence for cells killing IR162 cells.

However, interferon enhancement of natural killer cell activity has been extensively documented (3,18,20). Since we were primarily interested in the *in vivo* effects of spleen cells, a procedure was chosen which has been shown to induce lymphokines, including interferon, in rats (17). Both single high dose (100mg) and repeated low dose (5mg) challenges with OT inhibited tumor development. Thus lymphokine induction was shown to be effective as nonspecific immunotherapy. Therefore, there is at least suggestive evidence that the cell type responsible for the observed nonspecific immunotherapy of the rat myeloma may be the natural killer cell.

Since the tumor cells were susceptible to antibody mediated killing *in vitro*, they might be susceptible to specific immunotherapy. Several approaches were tried. First, spleen cells from donor rats at the same stage of tumor development as the recipients (day 8) were used. Although tumor cells were found in the spleen later in the course of the disease, tumor cells are not present at this early stage (11). If immunity were developing during tumor growth, concomitant immunity, we might expect to detect increased tumor inhibition. Instead we observed enhanced tumor growth. Attempts to find spleen cells capable of killing tumor cells *in vitro* during the first two weeks of tumor development were also unsuccessful.

We therefore postulate the development of immunosuppressive spleen cells early in myeloma development. Myelomas have been documented to be immunosuppressive (7); but it was somewhat surprising to find the suppression so early in tumor development. Perhaps that helps to explain the spontaneous nature of the rat myelomas and how readily they may be transplanted. Mouse myelomas, in contrast, must be induced by the intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane) and generally such an injection is used to "prime" mice for tumor transplantation. This appears to be due to the immunosuppressive activity of the pristane (1). The ability of the rat myeloma to suppress

the immune system early in its development could explain the difference between the two models.

Two methods of tumor immunization were tried. Rats were given a low tumor inoculum so that only about half the animals developed tumors. Then the tumor free rats were compared to rats of the same age which had never been exposed to IR162. The second tumor inoculum was also low. The rats which had been previously exposed to the tumor showed no increased resistance to tumor development; they did not appear to have developed any specific immunity. Other Lou/M rats were immunized with killed cultured tumor cells. Cultured cells were used because they had been cloned and were known to be only IR162 cells (10). Tumors do contain other cell types (15). The sporadic nature of the antibody forming response probably indicates that immunization was not optimum. However, some rats developed antibodies capable of killing the tumor cells. Therefore, the tumor cells do contain antigens capable of eliciting an immune response in syngeneic rats, and those cells are susceptible to antibody-complement mediated killing.

In vitro tests thus demonstrate a potential for the development of protocols for specific immunotherapy of the IR162 rat myeloma, although optimum immunization protocols have not been obtained. However, the IR162 tumor model is definitely susceptible to nonspecific immunotherapy *in vivo*. Since the tumor cells can be used to evaluate drug action *in vitro*, and they themselves are not particularly sensitive to a number of immunomodulatory drugs (9), this provides a promising model for *in vivo* trials of immunomodulators. One could be relatively certain that the action of such drugs was on the immune system rather than directly on the tumor cells. Therefore, results should reflect the response of the host to the drug rather than the sensitivity of individual tumors to those compounds.

Acknowledgments

Supported in part by a Saint Mary's College Faculty Research grant. We also wish to acknowledge the participation of Lisa Anselmino, Roberta Adams, Elizabeth Spinuzza, Alexis Swedish, and Mary Ososkie in various aspects of this work.

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