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Preferential Depression of IgM Producing Immunocytes during Mastocytoma-induced Immunosuppression (40058)

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Mastocytoma cells derived initially from mast cells of DBA/2 mice are markedly immunosuppressive. Immunologic impairment occurs when relatively small numbers of these tumor cells are added to normal spleen cells immunized *in vitro* with sheep red blood cells (SRBC) or alloantigens (1, 2). Cell-free extracts and/or ascites fluid from tumor-bearing animals also are immunosuppressive (3–4). *In vivo* studies have shown that immunologic impairment occurs in mastocytoma-bearing mice in the late stages of tumor progression (6). However, early after implantation of mastocytoma cells there is no significant suppression of immune responsiveness and sometimes even an enhancement.

Antibody formation to SRBC in mastocytoma-bearing mice, as well as in spleen cell cultures treated with mastocytoma cells or extracts, has been measured only at the level of 19S IgM production. Since it is possible that the tumor cells may affect differently immunocytes involved in a primary vs. secondary immune response it seemed of value to determine the effects of mastocytoma on 7S IgG antibody producing cells. In the present study the effects of the immunosuppressive mastocytoma cells and cell-free extracts on appearance of IgM vs. IgG antibody producing cells after primary or secondary immunization were examined.

Methods and materials. Animals. Male DBA/2 mice obtained from Cumberland View Farms, Clinton, TN, were used for these experiments. The animals were approximately 6–8 weeks of age and weighed 18–20 g when used. They were fed Purina mouse pellets and water *ad libitum.*

Tumor cells. Mastocytoma tumor cells, designated PB815X, were obtained originally from the American Type Culture Collection, Rockville, MD. They were passaged through DBA/2 mice by intraperitoneal (ip) injection every other week.

Antigen. SRBC were obtained in Alsever's solution from Cappel Laboratories, Downingtown, PA, and washed several times with minimum essential medium (MEM) purchased from Microbiological Associates, Bethesda, MD. Mice were injected by intravenous (iv) inoculation of 0.2 ml of a 10% suspension of freshly washed erythrocytes.

Antibody assay. Both the direct and indirect assays for hemolytic plaque forming cells (PFCs) to the SRBCs were performed with the spleen cell suspensions essentially as described earlier (1, 3). For the direct assay varying numbers of washed spleen cells from control and mastocytoma-bearing and/or ascites fluid injected mice were tested by the localized hemolytic gel assay essentially as described initially by Jerne *et al.* (1, 3). For this purpose 0.1 ml of a cell suspension was added to 2.0 ml melted Difco agar (40°) containing an 0.1 ml inoculum of a 1% suspension of RBCs. The agar cell mixture was poured carefully onto the surface of a previously prepared 60 mm diameter Petri plate containing a base layer of solidified agar. After the upper layer solidified the plates were incubated for 1 hr at 37° and then treated with approximately 5 ml of a 1:15 dilution of sterile guinea pig serum as the source of complement. The plates were incubated further for one to 2 hr until zones of hemolysis developed. These were considered due to direct 19S IgM PFCs. For indirect assay a facilitation procedure was utilized with antimouse gamma globulin serum (7, 8). To each agar plate was added 0.1 ml of a 1:100 or 1:200 dilution of rabbit antimouse 7S IgG serum with specificity to the heavy chain. The additional PFCs which developed on such plates were considered due to 7S IgG antibody (i.e., the numbers of direct PFCs were subtracted from the total number after the additional plaques appeared). In all cases the numbers of anti SRBC PFCs were determined on duplicate plates containing spleen cells from a single animal and the average

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number of PFCs was calculated for each mouse spleen.

Ascites fluid. Mice were injected by ip inoculation of 10^6 tumor cells. Fourteen to fifteen days later the ascites fluid (AF) was obtained from individual mice by aspiration of the peritoneum with a needle and syringe. Pooled AF was clarified by centrifugation at 2000*g* at 4°. The cell-free supernatants were frozen in small aliquots at −20°. The AF was thawed and warmed to 37° immediately before ip injection into test mice.

Results. Mice bearing mastocytoma tumors showed significant suppression of the IgM PFC response after either primary or secondary immunization with SRBC (Table I). For example, whereas normal mice developed many splenic IgM PFCs, with peak numbers on day 5 after immunization, mice given 10^6 mastocytoma cells ip 15 days or longer before primary immunization showed a depressed response. This suppression was evident on each day of assay. Although the peak response was about two thirds lower than that occurring in nontumor bearing control mice, the day of peak response still occurred on the same day, i.e., day five.

The indirect 7S IgG PFCs appeared at a relatively slower rate in both normal and tumor-bearing animals after primary immunization, with the peak on days 6 to 8. At these times, however, there were two to three times as many IgG as compared to IgM PFCs. The mastocytoma-bearing mice had essentially the same number of 7S IgG PFCs as did control mice. During the secondary immune response there was also no significant depression of the IgG PFC response (Table I). For these experiments mice were primed with sheep RBCs and injected with mastocytoma cells 15 days later. All mice received a second injection of sheep erythrocytes one month after the first injection. There was a marked depression of appearance of IgM PFCs in the spleen of these animals (approximately 75% fewer PFCs on the peak day as compared to control animals). In contrast, little if any effect on the IgG PFC response was evident in the tumor bearing animals (Table I). In other experiments, not shown, little if any effect was evident on the IgG PFC response in mice primed with SRBC at different times prior to injection of mastocytoma cells and secondary immunization.

Since it was difficult to control the size of the tumors in the mice at various times after implantation of mastocytoma cells, and since the absolute numbers of tumor cells per mouse was variable, addtional experiments were carried out in which known amounts of AF harvested from tumor-bearing mice were injected prior to primary or secondary immunization with SRBC. As can be seen in Table II, mice injected with 0.5 ml AF before primary challenge immunization with SRBC showed a marked impairment of their expected PFC response. However, this suppression was evident mainly at the level of IgM PFCs. The appearance of 7S IgG PFCs was

TABLE I. HEMOLYTIC ANTIBODY PLAQUE RESPONSE IN THE SPLEEN OF CONTROL AND MASTOCYTOMA-BEARING MICE AFTER PRIMARY VS. SECONDARY IMMUNIZATION WITH SRBC.

Anti-SRBC response on day after immunization[a]	Mouse group tested			
	Normal controls		Mastocytoma injected[b]	
	IgM PFCs	IgG PFCs[c]	IgM PFCs	IgG PFCs[c]
Primary +4 days	64,500	13,600	12,300	15,700
+5	97,300	58,960	38,700	65,400
+6	71,500	165,000	31,500	150,000
+8	40,350	130,500	22,300	125,000
Secondary +3	9,750	73,100	1,500	46,500
+4	12,600	195,000	3,750	170,000
+5	11,700	210,000	2,130	195,000
+7	5,650	97,500	1,600	112,000

[a] Groups of five to eight mice given either one or two iv injections of 4×10^8 SRBCs; secondary injected mice given booster immunization 30 days after primary injection.

[b] Mice injected ip with 10^6 mastocytoma cells 14 days before either primary or secondary immunization.

[c] Indirect 7S IgG PFCs detected by facilitation procedure with antiglobulin serum.

TABLE II. CYTOKINETICS OF APPEARANCE OF HEMOLYTIC PFCs IN THE SPLEEN DURING PRIMARY RESPONSE TO SRBCs BY NORMAL MICE AND MICE INJECTED ip WITH ASCITES FLUID FROM MASTOCYTOMA-BEARING MICE.

Time in days after primary immunization[a]	Mouse group tested			
	Normal controls		AF injected[b]	
	IgM PFCs[a]	IgG PFCs[d]	IgM PFCs[c]	IgG PFCs[d]
0	<100	<100	<100	<100
−3	11,920	3,436	8,760	2,278
−5	86,750	29,750	15,750	21,500
−7	59,340	187,300	10,300	174,000
−10	21,150	145,500	8,450	130,000
−15	7,300	37,400	2,150	35,300

[a] Groups of five to six mice injected iv with 4×10^8 SRBCs and assayed on day indicated for PFCs.
[b] Mice treated by ip injection of 0.5 ml A.F. on day −1 and 0 relative to day of immunization with SRBC.
[c] Direct PFCs for high efficiency 19S hemagglutinin antibody.
[d] Indirect PFCs detected by facilitation procedure with antiglobulin serum.

essentially normal in these AF-treated mice (Table II). The day of peak response of both classes of PFCs was essentially the same, regardless of whether or not the mice were treated with AF.

Normal mice primed with SRBC and then given a second injection of erythrocytes developed a markedly enhanced IgG PFC response. As is evident in Table III, mice injected prior to secondary immunization with AF showed a depression of the IgM but not the IgG PFC response, similar to the suppression occurring in mice bearing mastocytoma tumors. During the secondary resonse control mice showed a rapid appearance of IgG PFCs, with peak numbers 4 days after booster stimulation. At this time there were about 25 times as many IgG PFCs as IgM PFCs in the control animals. Mice injected with AF before secondary immunization showed esentially a similar IgG PFC response, but only about one third the IgM response, as compared to controls. Thus on the peak day of the secondary response there were about 80 times more IgG PFCs than IgM PFCs in AF treated mice. On days 6 to 8, when the numbers of IgM PFCs were decreasing rapidly, the numbers of IgG PFCs remained relatively elevated in the spleen of both control and AF-treated mice.

Discussion and conclusion. The results of these experiments showed that mastocytoma-bearing mice depressed in their ability to express a normal IgM antibody response to SRBC are, nevertheless, capable of a normal 7S IgG PFC response. Similar results were obtained for both the primary and secondary antibody response. Mice given a single injection of SRBC showed a rapid appearance of specific IgM hemolytic PFCs, with peak numbers on days 4 to 5 after immunization. At this time some IgG PFCs were evident, especially since the mice were given an optimum dose of RBC. The peak IgG PFC response occurred several days later. Mice actively bearing a mastocytoma tumor showed a marked depression of the IgM response, but only a slight, if any, suppression of the IgG PFC response. This was evident when mice were inoculated with 10^6 tumor cells approximately 2 weeks before primary immunization.

When RBC primed mice were given mastocytoma cells two weeks before a secondary immunization, there was a similar difference in the IgM vs. IgG antibody response. The major difference between primary and secondary responses was the appearance of large numbers of IgG PFCs, with peak responses several days earlier than during the primary response. Mice injected with mastocytoma cells two weeks before secondary immunization had a marked depression of the 19S IgM antibody response, but no consistent alteration of response, despite the fact peak days were similar to controls.

In order to investigate whether suppression was due to the immunosuppressive factor(s) found in previous studies, AF from mastocytoma-bearing mice was injected into normal animals prior to primary or secondary immunization with SRBC. Similar to the findings with the mastocytoma-bearing mice, AF treatment resulted in a preferential sup-

TABLE III. CYTOKINETICS OF THE SECONDARY HEMOLYTIC PFC RESPONSE IN THE SPLEEN OF NORMAL MICE AND MICE INJECTED WITH A.F. FROM MASTOCYTOMA-BEARING MICE IMMEDIATELY BEFORE A BOOSTER INJECTION WITH SHEEP RBCs.

Time in days after secondary immunization[a]	Mouse group tested			
	Normal controls		AF injected[b]	
	IgM PFCs	IgG PFCs[c]	IgM PFCs	IgG PFCs[c]
0	1,250	650	975	590
+2	5,970	21,350	2,360	17,340
+4	10,340	253,000	3,750	240,400
+6	8,730	195,000	1,530	210,700
+8	3,650	76,300	1,150	79,500
+10	1,950	12,500	738	23,500
+15	1,235	9,350	650	12,600

[a] Groups of five to six mice first injected iv with 4×10^8 SRBCs and then given a secondary injection 15 days later with same dose of erythrocytes on indicated day before assay for splenic PFCs.

[b] Mice injected i.p. with 0.5 ml AF on day −1 and 0 before secondary immunization with SRBC.

[c] Indirect 7S IgG PFCs detected by facilitation procedure with antiglobulin serum.

pression of the IgM as compared to the IgG response. These results point to the need for examining several parameters of the immune response, even to the same antigen, in order to determine mechanisms. The lack of a significant depression of IgG PFCs after secondary immunization suggests that the mastocytoma immunosuppressive factor(s) preferentially affects those immunocytes forming macromolecular hemolytic antibodies, i.e., 19S IgM hemolysis. On the other hand those immunocytes which develop later in the primary immune response and in larger numbers during the secondary response to SRBCs and secrete 7S hemolysins are essentially unaffected. These cells, presumably B-lymphocytes, require T-cells for antibody formation.

Results of this study appear to be at variance with earlier studies from this laboratory in which it was shown that "educated" T cells, when added to immunosuppressed spleen cell suspensions from mastocytoma bearing animals, restored immune responsiveness. It seemed likely from that study that a major immunologic defect caused by mastocytomas, at least in terms of responsiveness to SRBC, was related to helper T cell functions (9). If this were so, then mastocytoma treated mice should show a preferential impairment of the IgG rather than the IgM PFC response, since the "switch" to IgG antibody formation is thought to depend on T cell production. However, differences between *in vivo* vs. *in vitro* responses may explain this apparent inconsistency. Factors which influence the immune response *in vivo* may not be important or may be lacking during *in vitro* conditions. Also, helper T cell activity for anti-SRBC responses may be expressed through different mechanisms for primary vs. secondary responses.

The results of the present studies suggesting that "conversion" of IgM to IgG antibody responsiveness in vivo is less affected than formation of IgM antibody per se also contrasts with the widely accepted view that IgG antibody formation depends upon a preceding IgM antibody response (10). However, it is not yet clear whether separate classes of precursor cells exist for these two cell populations. Regardless of the mechanism involved, it seems apparent from the results of this study that mastocytoma-bearing mice per se as well as mice treated with AF from tumor-bearing animals show a preferential depression of the IgM antibody response as compared to the IgG response.

Summary. Immunologic impairment occurs in mice bearing mastocytoma tumors or injected with cell free extracts and/or ascites fluids from the tumor bearing animals. In the present study it was found that the 19S IgM antibody response to sheep red blood cells as assessed at the individual hemolytic plaque forming cell level was preferentially affected. Whereas there were 60–80% fewer IgM PFCs in mice given a primary injection with sheep erythrocytes, there was only a slight effect on appearance of indirect 7S IgG plaque forming cells. Furthermore, mice primed with

sheep red blood cells and then implanted with mastocytoma cells showed a marked impairment of the secondary IgM response but no depression of the IgG response. Injection of cell free ascites fluid derived from mastocytoma-bearing mice into normal recipient mice before primary or secondary immunization with sheep red cells resulted in marked depression only of the IgM response. These results indicate that mastocytoma affects preferentially in immunocytes producing IgM hemolytic antibody and not those involved in formation of 7S antibody.

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