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Prolonged Adjuvant Stimulation in Germ-Free BALB/c Mice: Development of Plasma Cell Neoplasia

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Summary. BALB/c mice develop a high incidence (60–80 per cent) of plasma cell tumour (PCT) following the introduction of mineral oil (MO) into the peritoneal cavity. Germ-free (GF) mice have a less developed lymphoreticular system, including a decreased number of plasma cells, than their conventional (CONV) counterparts. When GF BALB/c mice were injected i.p. with mineral oil they failed to develop the expected incidence of PCT. Only two of thirty-three GF mice (6 per cent) developed PCT, while twenty-four of thirty-one ex-GF (77 per cent) and twenty-eight of forty CONV BALB/c (70 per cent) developed PCT. The ex-GF and CONV mice had average times for PCT diagnosis of 11·3 and 11·5 months, but both GF tumours were discovered 18 months after mineral oil injection. Three groups of GF mice received three different sterile protein antigens along with MO and no PCT developed in these mice; in the GF group receiving MO alone, both PCT arose. The GF mice in this experiment did develop a high incidence (80 per cent) of lymphoreticular neoplasms of a type more primitive than PCT. This experiment suggests the importance of microbial flora in the development and differentiation of the plasma cells which respond to a carcinogenic stimulus in a genetically susceptible host.

INTRODUCTION

Plasma cell neoplasms of humans and mice have been used in the study of specific aspects of the immunoglobulins produced by these interesting malignancies (Cohen and Milstein, 1967; Potter, 1967) and for comparison of the tumour-secreted proteins with the products of the normal immune response (Hill, Delaney, Fellows and Lebowitz, 1966; Wikler, Kohler, Shinoda and Putnam, 1969). Recently these tumours have been shown to produce immunoglobulins with antibody activity (Metzger, 1967; Eisen, Little, Osterland and Simms, 1967; Cohn, 1967; Potter and Leon, 1968; Eisen, Simms and Potter, 1968; Metzger and Potter, 1968). While the normal immune response, leading to the production of circulating antibodies has been intensively studied, little is known of the pathogenesis of plasma cell tumours.

In mice plasma cell tumours (PCT) are induced in the BALB/c strain by the introduction of plastics (Merwin and Algire, 1959) or mineral oil (Potter and Robertson, 1960) into the peritoneal cavity. The tumours appear months later following the chronic interaction between host tissues and foreign material. Lymphoreticular tumours (plasma cell leukaemias) which are capable of secreting immunoglobulin products are also induced in this

strain by the inoculation of sub-cellular extracts of mouse PCT (Ebbesen, Rask-Nielsen and McIntire, 1968; Rask-Nielsen, McIntire and Ebbesen, 1968). The spontaneous disease has been described rarely in mice and only in a few strains (Rask-Nielsen and Gormsen, 1951; Dunn, 1957).

Germ-free (GF) mice are known to have a less developed immune system than their conventional (CONV) counterparts (Bauer, Horowitz, Levenson and Popper, 1963; Olson and Wostmann, 1966a, b) and this includes significantly fewer plasma cells and precursors, fewer lymphocytes, and a lack of germinal centres in all lymphoid tissues. Along with the hypocellular reticuloendothelial system is a low serum immunoglobulin (Ig) level (Wostmann, 1961; Sell and Fahey, 1964) and a delayed responsiveness to some forms of antigenic stimulation but a normal response to others (Bauer, Horowitz, Watkins and Popper, 1964; Sell, 1965; Olson and Wostmann, 1966b).

This study was undertaken to demonstrate the effect of the GF environment, with the resultant decreased number of potential antibody cells and absence of viable bacterial flora and other conventionally potential antigens, on the induction of PCT in mice.

MATERIALS AND METHODS

Mice

BALB/c AnN mice were introduced into the GF environment by Casearian section and foster-nursed on Lobund, outbred Swiss mice. They were maintained in a Reyniers type stainless steel isolator, kept five mice to a cage, and fed sterilized diet (Cohen, Newton, Cherry and Updyke, 1963). Routine bacterial cultures of the GF unit were taken every 2 weeks. The ex-GF mice were born and reared under GF conditions until 1–2 months of age when they were removed and placed in a conventional animal room environment; CONV BALB/c AnN mice were obtained from the Animal Production Section, National Institutes of Health. They were housed four to eight per cage, fed non-sterile lab chow and water *ad libitum*. All mice in this experiment were female; they were given their first mineral oil injection at 1–2 months of age and received two additional injections 2 and 4 months after the first. All injections were given intraperitoneally with a volume of 0·5 ml.

Mineral oil

Bayol F from Esso Standard Oil Co. of New Jersey was autoclaved for 60 minutes at 256°F. Some was taken into the GF unit and the remainder was used for the ex-GF and CONV BALB/c mice, stored in a cotton-stoppered flask at room temperature.

Antigens

Dinitrophenyl-ovalbumin (DNP-ovalbumin), Keyhole Limpet haemocyanin and horse ferritin (kindly provided by Dr Stewart Sell) were passed through a Seitz filter and were taken into the GF isolator in sterile vials using peracetic acid (Horton and Hickey, 1961). DNP-ovalbumin was injected intraperitoneally in three doses (0·75, 1·5 and 0·75 mg) given 4 weeks apart. Haemocyanin and ferritin were both given in a single intraperitoneal injection of 1 mg. All antigens were given initially at the time of the third mineral oil inoculation.

Pathology

Tissues were fixed with Tellesnickzky's fluid and stained with haematoxylin and eosin.

Serum protein studies

Electrophoresis and immunoelectrophoresis were carried out in agar gel at pH 8·2 (McIntire and Potter, 1964). Antisera to the mouse immunoglobulin types were made in rabbits using purified mouse myeloma proteins as antigens (Potter, 1967). Testing for precipitating antibodies was by double diffusion (Ouchterlony method) in agar gel pH 8·2.

Statistical analysis was by the method of χ^2.

RESULTS

Forty-five GF mice lived longer than 6 months after the first injection of mineral oil. Those that were not killed and autopsied as they developed signs of imminent death were killed at 2 years of age. Tissue sections for histological study were obtained on thirty-three mice and the results are shown in Table 1. Lympho-reticular neoplasms were found in twenty-six mice (80 per cent) and the majority of these were pleomorphic reticulum cell sarcomas (McIntire and Law, 1967; Dunn and Deringer, 1968) containing plasma cells, histiocytes, reticulum cells and lymphocytes. Monomorphic reticulum cell sarcomas (RCS) were found in seven animals. Only two mice developed PCT and one a lymphocytic neoplasm. These tumours were found in mice from 12–24 months after mineral oil injection, but the average time to necropsy was 17·5 months. Both PCT were discovered in the 18th month of the experiment when the mice were 20 months of age. Ascites samples were smeared and studied for cell composition whenever possible. The two mice which developed PCT were the only two that had malignant plasma cells in the ascites; the rest had varying degrees of cellularity with histiocytes or lymphocytes predominating.

TABLE 1

NEOPLASMS FOUND IN BALB/C MICE FOLLOWING INTRAPERITONEAL INJECTION OF ADJUVANT

Tumour type	Germ-free	Ex-germ-free	Conventional
Plasma cell tumour	2 (18)*	24 (11·3)*	28 (11·5)*
Pleomorphic reticulum cell sarcoma	16 (21)	3 (14·7)	4 (11·5)
Monomorphic reticulum cell sarcoma	7 (20)	1 (12)	1 (9)
Mixed (pleo- and monomorphic reticulum cell sarcoma)		2 (18·5)	
Lymphocytic neoplasm	1 (23)		
Pulmonary adenoma	2 (20)		2 (17)
Myoepithelioma	1	1	1
Uterine adenoma	1		
Reticular hyperplasia (no tumour)	3		4
Total	33	31	40

*Average time of diagnosis in months following injection of mineral oil.

The tumour incidence observed for the forty ex-GF BALB/c female mice was strikingly different from the GF mice, as shown in Table 1. There was histological evidence of plasma cell neoplasia in twenty-four out of thirty-one autopsies (77 per cent); only three had pleomorphic RCS and one had monomorphic RCS. There were two mice which developed both a monomorphic and pleomorphic RCS in different areas. The average latent period following mineral oil inoculation for PCT was 11·3 months, for pleomorphic RCS 14·7 months, and for the mixed tumours 18·5 months. Nine ex-GF mice were not

autopsied; two of these probably had PCT since malignant plasma cells were found in the ascites, three had no gross evidence of tumour up to 22 months when they were lost and four were found dead between the 10th and 15th month and may have had a reticular neoplasm.

A group of CONV BALB/c mice showed results similar to ex-GF mice (Table 1). Seventy per cent developed PCT at an average onset of 11·5 months after mineral oil, 10 per cent developed a pleomorphic RCS also at an average 11·5 months and one mouse developed monomorphic RCS at 9 months. The difference in incidence of PCT between the GF and non-GF (ex-GF and CONV) mice was statistically significant ($P < 0{\cdot}001$).

Attempts were made to transplant all PCT and the majority grew progressively in syngeneic hosts; both PCT in GF mice, twenty in ex-GF and twenty-four of the PCT in CONV mice were transplantable. The pleomorphic RCS also grew in transplant from all three groups; fourteen of the GF, one of the ex-GF and three of the CONV. All of the monomorphic RCS grew in transplant as did the lymphocytic neoplasm and one of the two mixed tumours.

TABLE 2

PATHOLOGICAL FINDINGS IN GERM-FREE MICE ACCORDING TO ANTIGENIC STIMULATION

Pathology	No. of mice in each antigen group			
	DNP-ovalbumin	Haemocyanin	Ferritin	None
Plasma cell tumour				2
Pleomorphic reticulum cell sarcoma	5	4	5	2
Monomorphic reticulum cell sarcoma	3		1	3
Lymphocytic neoplasm		1		
Pulmonary adenoma	1		1	1
Uterine adenoma				1
Myoepithelioma		1		
Reticular hyperplasia (no tumour)	1			1
No autopsy	5	4	2	1
Total	15	10	9	11

To study the effect of antigenic stimulation along with mineral oil adjuvant in the induction of PCT in GF mice, three groups were given different protein antigens. The pathologic findings are in Table 2. The number of mice in each group is small, but the striking feature is that both mice developing PCT were in the non-antigenically stimulated group (in different cages) and none of the mice receiving protein antigens developed PCT. This difference between antigen-treated and the non-immunized GF mice with regard to PCT development is significant ($P < 0{\cdot}02$).

Of the five unautopsied mice in the DNP-ovalbumin group, four had ascites not containing malignant plasma cells. The four unautopsied mice in the haemocyanin group included two where post-mortem tissues were of a quality adequate to rule out PCT, and a third had ascites not containing malignant plasma cells.

Because of the difficulty and the mortality associated with the bleeding of GF mice, antibody production was not studied until the time of death. The mice immunized with DNP-ovalbumin were given the first injection 4 months after the first mineral oil and then received two subsequent injections at 4-week intervals; all injections were given i.p. in aqueous solution. Ten mice were bled at the time of autopsy which was an average 17·2

months after the immunization; six of ten showed precipitating activity against ovalbumin and eight of ten with DNP-bovine serum albumin. The mice given haemocyanin and ferritin were given a single i.p. injection 4 months after the first mineral oil. Thirteen mice were bled in these two groups at an average 16·5 months after immunization and none showed precipitating activity against the appropriate antigen.

TABLE 3

IMMUNOGLOBULIN TYPES PRODUCED BY PLASMA CELL TUMOURS ARISING IN BALB/C MICE

	γA	γG	γH	γF	γM	BJ*	None	Total
Germ-free	1 (1)†				1			2
Ex-germ-free	13 (5)	2 (1)	3	2	1	1	2	24
Conventional	16 (3)	2 (1)	2		1	4	3	28

* Bence Jones proteins were all κ type.
† Numbers in parentheses are those that also had Bence Jones proteinuria.

The types of immunoglobulins produced by PCT (Table 3) in the ex-GF and CONV mice is the expected pattern, seen with other PCT-induction experiments with mineral oils in BALB/c mice. More than half of the tumours produced γA, an occasional tumour produced γM, almost 10 per cent produced only light chains and the same number produced no detectable specific immunoglobulin, the remaining tumours were divided among the 7S γ classes of the mouse (γF, γG, γH). In the GF mice, both PCT produced immunoglobulin: one γA and the other γM. All other tumours were carefully checked by immunoelectrophoretic screening in the original form and after establishment as a transplantable tumour and none was found to produce monoclonal immunoglobulin elevation.

DISCUSSION

The first difference observed between GF and ex-GF or CONV mice is the delay in tumour development in GF. At a time (16 months) when almost all the PCTs had developed and 85–90 per cent of the CONV and ex-GF mice had died, only 15 per cent of the GF mice had come to autopsy and none had PCT. This delay in lymphoreticular tumour development could be related to the fact that GF mice have far fewer blast cells in lymph nodes than do CONV mice (Olson and Wostmann, 1966a). When the incidence of reticular tumours is compared at the end of 24 months the total in GF mice is 79 per cent, in ex-GF it is 97 per cent and in CONV it is 82 per cent. There is not a significant difference among the different groups in terms of total reticular neoplasms, but there are distinct differences in the types of neoplasms. CONV and ex-GF mice had 70 and 78 per cent frequencies of PCT contrasted with only 6 per cent in GF mice. The pleomorphic RCS accounted for almost one-half of all the neoplasms in GF mice, but only a 10 per cent frequency in the ex-GF and CONV mice. Twenty-one per cent of GF pathology showed the monomorphic RCS but only 3 per cent of the ex-GF and CONV had the same. The development of reticular neoplasms in GF mice following intraperitoneal adjuvant stimulation could be analagous to induction of plasma cell neoplasia in CONV mice with a greatly diminished concurrent antigenic stimulation. Or the reticular neoplasms seen here in GF mice could be the routine fate for BALB/c mice when removed from other tumourigenic stimuli and allowed to live for 20–24 months and be unrelated to the intraperitoneal adjuvant. Regardless of the explanation, the fact remains that in the absence of normal microbial flora the induction of plasma cell neoplasms in BALB/c mice is markedly reduced.

The addition of a foreign protein antigen did not change the pattern or time of tumour development among the groups of GF mice except that the absence of a specific injected antigen did appear to favour the development of PCT. The actual role of the various antigens is impossible to assess. Both haemocyanin and ferritin were given only as a single injection and by the time the mice were autopsied, none had detectable precipitating activity. DNP-ovalbumin in three injections resulted in precipitating antibody 13–20 months later in eight of ten mice. The two which failed to respond both had monomorphic RCS. Sera from mice with transplanted tumours did not show activity against the DNP-protein or ovalbumin antigen even when the mouse with the original tumour did show precipitating activity.

The ex-GF and CONV mice both had slightly more than half of the PCT producing γA type immunoglobulins, while the other half was divided among the remaining possibilities in a predictable pattern (Potter, 1967). No tumours were found to produce λ type light chains. The finding that γA was produced by one of two GF tumours is surprising since the γA response to antigenic stimulation in GF mice is very low or lacking (Asofsky and Hylton, 1969). The γA levels of the GF mice in this experiment were compared to the ex-GF and CONV mice which had tumours other than γA-producing and found to be only one-quarter as high. The γM-producing tumour, rare in CONV mice ($\sim$4 per cent), is not so unexpected in the GF condition since γM has been shown to be the principal immunoglobulin synthesized in GF mice (Asofsky and Hylton, 1969). A deficiency of cells capable of γA response in GF mice might indicate a decreased number of plasma cells capable of malignant transformation if these are the cells in CONV BALB/c mice with the greatest predilection for becoming neoplastic.

It has been suggested that prolonged antigenic stimulation possibly of autoimmune origin might result in neoplasia of immunoglobulin-producing cells in humans (Osserman and Takatsuki, 1965; Talal, Sokoloff and Barth, 1967) and in mice (Schubert, Jobe and Cohn, 1968). The availability of self-antigens in the absence of normal bacterial flora was not sufficient stimulation for PCT formation in GF mice. Since in BALB/c mice the PCT arises within areas of chronic granulomatous reaction in close association with the bowel the gut bacteria could be a source of necessary stimulus for PCT induction. The bacteria could be acting to cause cellular damage resulting in the release of self-antigens or it could be providing bacterial antigens in an area of chronic immune reactivity. Bacterial antigenic stimulation might also be necessary for multiplication of a specific plasma cell population, i.e. those susceptible to plasma cell carcinogenesis. A deficiency of these susceptible plasma cells might result in a decrease in PCT and a corresponding increase in tumours of a less differentiated or precursor cell.

It appears that the presence of viable bacterial flora or some common viral organism is of considerable importance in the development of PCT in a host that is genetically susceptible to the induction of such tumours.

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