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INFLUENCE OF THE SPLEEN AND THYMUS ON IMMUNE RESPONSES IN AGEING MICE

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SUMMARY

Primary haemagglutinin responses to sheep red cells were depressed in aged C57Bl and (AKR × C57Bl)F_1 mice but not in aged C3H mice. Splenectomy depressed haemagglutinin responses in both young and old mice but subcutaneous spleen grafts failed to restore immune responsiveness of splenectomized mice to normal levels.

Multiple spleen or thymus grafts in C57Bl mice failed to prevent the age-related decline in responsiveness to sheep red cells as estimated either by the haemolytic plaque-forming cell response in the spleen or haemagglutinin titrations. Multiple spleen and thymus grafts also failed to improve pre-existing depressed responses in old age.

Although adult thymectomy causes a delayed depression in responsiveness to sheep red cells, depressed thymus function in old age does not appear to be a factor limiting immune responses to sheep red cells in aged mice.

INTRODUCTION

It is likely that there is a progressive decline with advancing age in the capacity to develop effective immune responses but few systematic investigations have been made to confirm this or to analyse the factors which might be involved in the development of reduced immunological competence.

Teller *et al.* (1964) reported that aged mice exhibited a diminished capacity to reject transplants of foreign tumour cells. Makinodan & Peterson (1964) demonstrated that ageing mice contained fewer cells in the spleen which were capable of being stimulated by sheep red cells to proliferate and produce haemagglutinins, when injected into irradiated recipients; and Miller (1965) noted a fall with age in the number of background cells in the spleen capable of forming haemolytic plaques with sheep red cells using the Jerne agar plate technique. More recently Albright & Makinodan (1966) showed that not only was there a reduction with age in the number of spleen cells capable of being stimulated by sheep red

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cells, but those cells which were present were capable only of a limited degree of antigen stimulated proliferation even when placed in the environment of a healthy young animal.

The present investigations were undertaken to analyse the response with increasing age of various mouse strains to a single antigen (sheep red cells) and to investigate the role played by the spleen and thymus in age-related changes in immune responsiveness.

MATERIALS AND METHODS

Mice

Mice used were males and females of the inbred strains C57Bl, C3H and the F_1 hybrid strain (AKR × C57Bl)F_1 which are maintained in this Institute. Mice were housed in metal boxes with sawdust bedding and were fed dog pellets and water *ad libitum* with supplemental greens and carrots.

Immunization procedure

For the investigation of haemagglutinin responses all mice, regardless of body weight, received an intraperitoneal injection of 0·2 ml of 20% sheep red cells in normal saline. For the analysis of haemolysin and haemolysin plaque-forming cell responses, all mice received an intraperitoneal injection of 0·1 ml of 30% sheep red cells.

Haemagglutination titrations

Mice were bled from the tail vessels on days 3, 5, 7, 10 and 14 after immunization and the sera either titrated the same day or stored at −20°C before titration. Doubling dilutions of serum were made in plastic trays using 1% pooled rabbit serum in normal saline as the diluting fluid. Equal volumes (0·25 ml) of 2% sheep red cells in normal saline were added, and the trays shaken and allowed to stand undisturbed at room temperature for 2 hr before haemagglutination patterns were read. The last tube showing macroscopically visible haemagglutination of some of the cells was taken as the end point of the titration. Parallel studies confirmed that this end point corresponded to the end point of haemagglutination as determined by microscopic examination.

Haemolysin plaque-forming cell titrations

The agar plaque technique of Jerne, Nordin & Henry (1963) was used for the detection of 19S haemolysin-producing cells. Spleen tissue from immunized mice was minced in Eisen's solution containing 15% foetal calf serum. Cell suspensions were allowed to stand at 4°C for 30 min to allow cell clumps to settle out. Agar plates containing sheep red cells and test cell suspensions were incubated at 37°C for 1 hr, then 4 ml of 1:10 guinea-pig serum were added and the plates incubated at 37°C for 30 min to develop the haemolytic plaques. All plates were stained with benzidine and the plaques were counted at ×20 magnifications.

Spleen and thymus grafting

Spleens or thymus glands were removed intact from donor 1-day-old C57Bl mice and immersed in sterile normal saline. Recipient mice were anaesthetized with Nembutal and each mouse was grafted subcutaneously with either twelve whole spleens or twelve whole thymus glands by gently inserting these organs in scattered pockets prepared by blunt

dissection in the subcutaneous tissues overlying the abdomen and thorax. Skin wounds were closed with Michel clips.

Histological examination

At the time of killing for plaque counts or on day 14 for the other studies, portions of the lymphoid organs were fixed in 10% Zenker's formalin. If the animal appeared diseased, sections were taken also of other relevant organs. Tissues were dehydrated, blocked in paraffin, sectioned at 5 μ and duplicate sections stained with Mayer's acid haematoxylin and eosin or with methyl green pyronin.

RESULTS

Age changes in haemagglutinin responses

Groups of eighteen C57Bl mice of both sexes aged 3, 9, 12, 15 and 18 months were studied for their haemagglutinin response to primary immunization with sheep red cells. The results obtained are shown in Fig. 1. Although there was some variation in antibody responses between individual members in each age group, a progressive decline with increasing age was noted in the mean capacity to produce haemagglutinin. This depressed responsiveness appeared to be more marked at day 5 after immunization than at days 10 or 14. Additional studies indicated that haemagglutinin responses in 1-week-old C57Bl mice were very low but rose significantly by the age of 1 month and at 2 months were equivalent to those in 3-month-old mice.

A similar degree of depression of haemagglutinin responses was noted in (AKR × C57Bl)F_1 mice when groups of 18-month-old mice were compared with groups of 2-month-old mice (Table 1). Mice of the strain (AKR × C57Bl)F_1 spontaneously develop a number of tumours, the most common of which are reticulum cell sarcoma and hepatoma (Metcalf, 1964). In mice with reticulum cell sarcoma the spleen becomes replaced progressively by tumour tissue and in the earliest stage of the disease only the lymphoid follicles of the spleen are replaced by neoplastic cells. In the old (AKR × C57Bl)F_1 mice analysed in the present series, twenty-six had either reticulum cell sarcoma or hepatoma. The haemagglutinin responses in mice with hepatoma were not significantly different from those of the disease-free old (AKR × C57Bl)F_1 mice but immune responses were significantly lower in mice with reticulum cell sarcoma (Table 1). Several of these mice were in the earliest stage of the disease and only the spleen lymphoid follicles appeared affected but these mice also exhibited greater depression of immune responses than in disease-free mice of the same age.

In contrast to the results with C57Bl and (AKR × C57Bl)F_1 mice, C3H mice exhibited no depression of immune responses when animals aged 16–19 months were compared with 2-month-old animals (Fig. 2). Four male mice in the aged C3H group were found to have advanced hepatoma but the immune responses in these tumour-bearing animals were identical with those of the other mice in this group.

Histological examination of lymphoid organs in aged mice

A comparative study was made of the lymphoid organs at day 14 after intraperitoneal immunization with 0·2 ml of 20% sheep red cells in forty-four 3-month-old and forty-four 18-month-old C57Bl mice. In the old mice the mean weights of the spleen and lymph nodes

(spleen 204±62 mg; six subcutaneous lymph nodes 69±15 mg; mesenteric node 74±44 mg) were heavier than in the young mice (spleen 130±30 mg; six subcutaneous lymph nodes 42±12 mg; mesenteric node 46±7 mg) whilst the thymus in the old mice was smaller

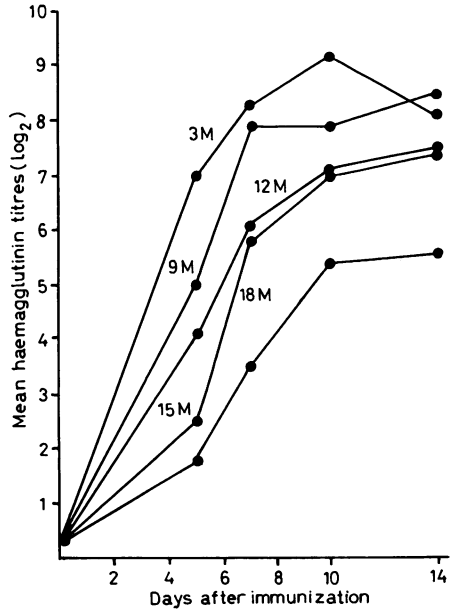

FIG. 1. Decline with advancing age in haemagglutinin response to primary immunization with sheep red cells in C57Bl mice. Age of mice indicated on each curve, e.g. 3M = 3 months old. Each point represents mean value for eighteen mice.

TABLE 1. Haemagglutinin responses in (AKR × C57Bl)F_1 mice

Age (months)	Diagnosis	No. of mice tested	Haemagglutinin titres (log_2) (± Standard Deviation)				
			D3	D5	D7	D10	D14
2	Normal	68	3·0±0·9	6·4±0·8	7·8±0·6	8·6±1·0	8·4±1·0
18–20	Normal	43	2·0±1·0	3·4±1·7	5·0±1·5	6·1±1·5	6·0±1·4
18–20	Reticulum cell sarcoma	19	1·5±0·8	3·1±1·5	3·4±2·1	4·3±2·1	4·0±2·1
18–20	Hepatoma	7	2·0±1·0	2·0±1·5	3·3±1·5	5·4±2·1	6·3±2·2

Titres in normal 18–20-month-old mice significantly lower ($P<0·01$) than in 2-month-old mice at days 5, 7, 10, 14 (t values = 12·0, 11·5, 10·2, 11·0 respectively).

Titres in 18–20-month-old mice with reticulum cell sarcoma significantly lower ($P<0·01$) than in normal 18–20 month-old mice at days 7, 10, 14 (t values = 3·1, 3·8, 3·7 respectively).

(19±7 mg) than in the young mice (40±6 mg). Similar results were obtained in comparative studies on the lymphoid organs in young and old (AKR × C57Bl)F_1 and C3H mice.

In all three strains the increased lymph node weights in the old mice were deceptive in suggesting increased lymphoid cellularity, for microscopic examination revealed that

cellularity of the lymphoid cortical areas of the lymph node was reduced and that germinal centres were infrequent and poorly developed in the older mice. The increased weights of these organs appeared to be due to an increase in the reticulo-endothelial cell elements in the medullary regions and, particularly in the case of the mesenteric nodes, to cystic dilatation of the medullary lymph spaces. In all three strains, the thymus in the older mice showed typical age involution with an overall reduction in the width of the lymphoid cortex.

Sections of the spleen in aged C57Bl and (AKR × C57Bl)F_1 mice exhibited the same percentage area of lymphoid follicles (19%) and the same number of follicles per unit area as in the young mice but the lymphoid follicles in old mice showed a considerable depletion in their content of small lymphocytes (Figs. 3 and 4). Germinal centres were only occasionally

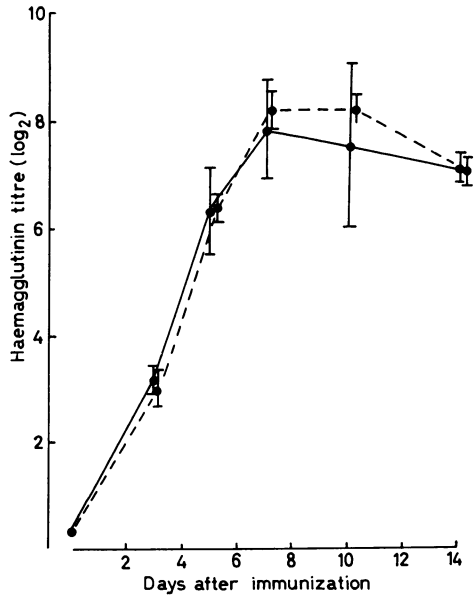

FIG. 2. Haemagglutinin responses in young and old C3H mice to primary immunization with sheep red cells. – – –, 2-month-old mice (thirteen); ——, 16–19-month-old mice (sixteen). Vertical bars are standard deviations of mean values.

visible in the spleen lymphoid follicles of old mice and most exhibited little mitotic activity. Frequently the entire cellular organization of the lymphoid follicle appeared to be disorganized. In contrast, the spleen lymphoid follicles in aged C3H mice were found to have a higher content of small lymphocytes and more active germinal centres than were present in the aged mice of the other two strains and these structural elements in the aged C3H spleen were almost as well developed as in young C3H mice.

Small groups of mice examined 4 and 5 days after immunization with sheep red cells suggested that the relative deficiency of spleen lymphoid follicle response in aged mice was even greater at this time after immunization than in the mice examined 14 days after immunization.

No obvious differences were seen between young and old spleens in the cellularity or architectural arrangement of the red pulp.

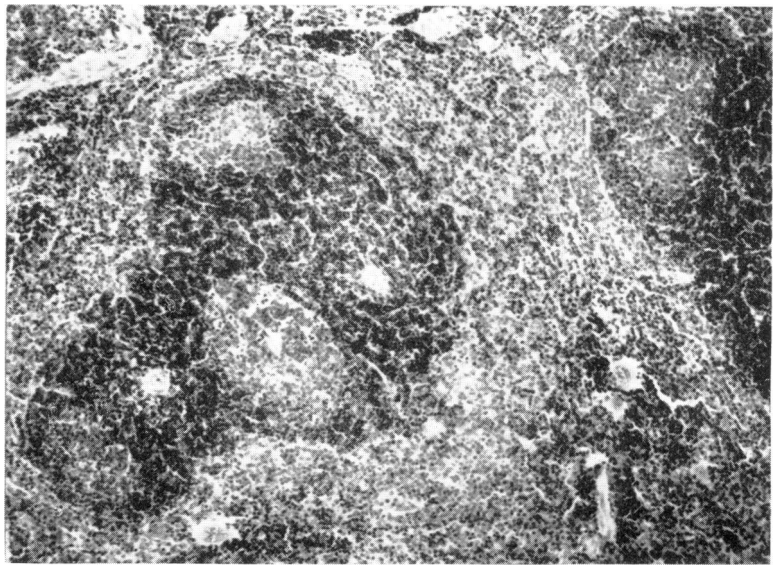

FIG. 3. Spleen lymphoid follicles in 3-month-old C57Bl mouse on day 14 after primary immunization with sheep red cells. Note tightly packed lymphoid cells in follicles and active germinal centres. H & E, × 125.

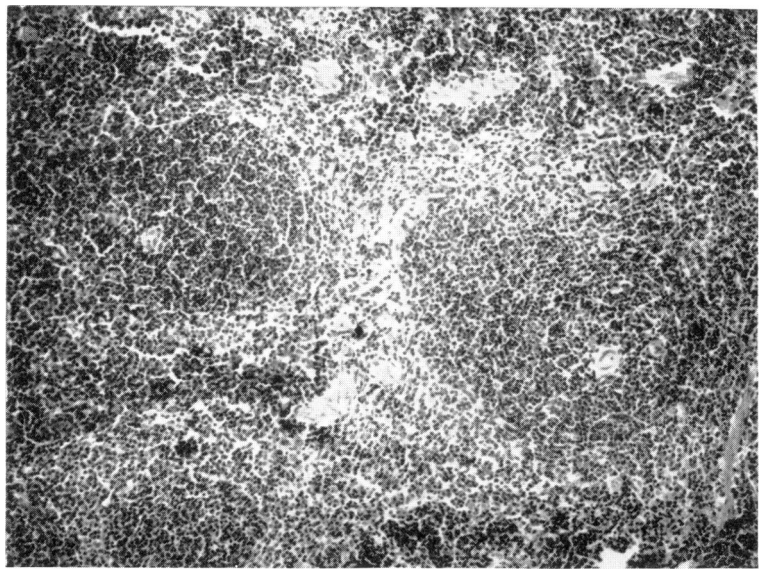

FIG. 4. Spleen lymphoid follicles in an 18-month-old C57Bl mouse on day 14 after primary immunization with sheep red cells. Note the loose acellular structure of the lymphoid follicles and the absence of germinal centres. H & E, × 125.

Effect of splenectomy and spleen grafting on haemagglutinin responses

In view of the finding that the most obvious difference between the lymphoid organs of young and old mice appeared to be the cellular depletion and disorganization of spleen lymphoid follicles, experiments were carried out to determine the effect of splenectomy in young and old mice on immune responses.

Two-month-old C57Bl and C3H mice were splenectomized or sham operated and, immediately after operation, were immunized with sheep red cells. Haemagglutinin responses in splenectomized mice were reduced by 85–90% when compared with those in sham operated animals (Table 2). Splenectomy or sham operation were also performed on groups of 18-month-old C57Bl and C3H mice immediately prior to immunization. Despite the depressed immune responses in sham operated C57Bl mice, splenectomy further reduced

TABLE 2. Effect of splenectomy on haemagglutinin responses in young and old C3H and C57Bl mice

Strain	Age at operation (months)	Type of mouse	No. of mice	Haemagglutinin titres (log_2) (± Standard Deviation)			
				D5	D7	D10*	D14
C3H	2	Sham splenectomy	12	6·6±0·8	8·0±0·3	8·5±0·8	8·4±0·6
		Splenectomy	12	4·4±0·6	6·6±0·9	6·5±1·0	7·2±0·5
C3H	18	Sham splenectomy	12	5·3±0·9	7·3±0·5	7·0±0·4	7·3±0·6
		Splenectomy	11	1·3±1·0	2·9±1·6	3·6±1·0	4·0±1·6
C57Bl	2	Sham splenectomy	15	6·2±1·0	8·6±1·0	9·2±0·8	8·0±0·7
		Splenectomy	23	2·3±1·0	5·1±1·5	5·6±1·2	5·6±1·9
	18	Sham splenectomy	24	2·2±1·0	3·8±1·6	5·5±1·3	5·3±1·0
		Splenectomy	25	1·5±0·8	2·2±1·0	2·8±1·2	2·8±1·1

* Day 10 titres in all groups of splenectomized and sham operated mice differ significantly on *t*-test $P<0·01$).

these immune responses by an amount similar to the reduction following splenectomy in young adult mice.

In further experiments, 2-month-old C57Bl mice were splenectomized or sham operated and, immediately afterwards, one group of splenectomized mice was grafted subcutaneously with two whole 1-day-old C57Bl spleens to each recipient. One month later all three groups were immunized with sheep red cells. Again immune responses in splenectomized mice were significantly reduced, particularly during the early phase of the response. Immune responses in the splenectomized mice carrying spleen grafts were not improved significantly by the spleen grafting procedure (Fig. 5), despite the fact that the total mass of spleen graft tissue (100 mg) approximated that of the normal spleen (150 mg). Tests made on splenectomized C57Bl mice which had been carrying spleen grafts for 2–3 months before immunization revealed that such mice still failed to exhibit immune responses significantly above those of splenectomized control groups. This unexpected failure of subcutaneous spleen grafts to restore immune responses was confirmed by similar experiments made on C3H mice splenectomized at 2 months of age and tested 1–2 months after grafting.

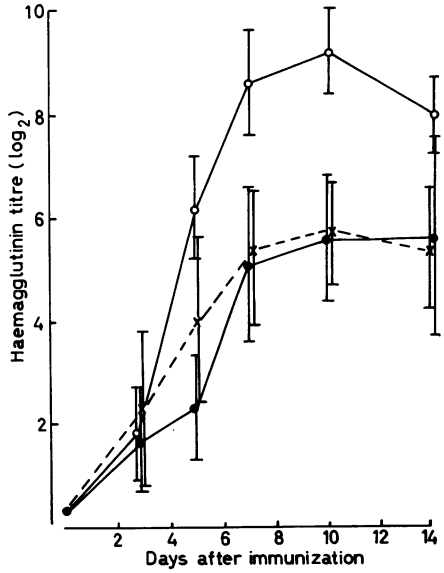

FIG. 5. Effect of splenectomy and spleen grafting in 2-month-old C57Bl mice on haemagglutinin responses to primary immunization with sheep red cells. ○, Sham-splenectomy (fifteen mice); ×, splenectomy+spleen grafts (fifteen mice); ●, splenectomy (twenty-three mice).

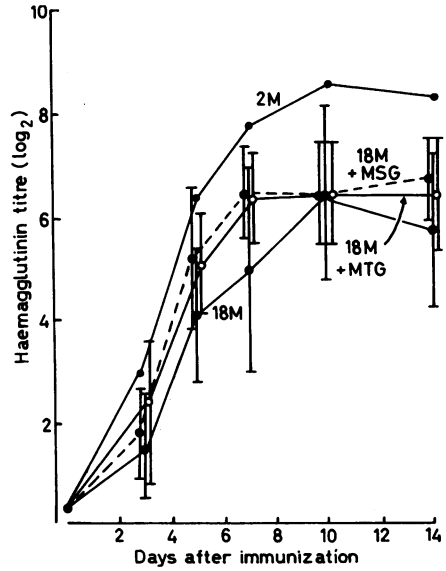

FIG. 6. Haemagglutinin responses in 18-month-old (AKR × C57Bl)F_1 mice grafted with multiple spleen grafts (18M + MSG) or multiple thymus grafts (18M + MTG) compared with responses in control 18-month-old (18M) and 2-month-old (2M) (AKR × C57Bl)F_1 mice. Vertical bars are standard deviations of mean values.

Effect of multiple spleen or thymus grafting on immune responses in aged mice

The effect was investigated in aged (AKR × C57Bl)F_1 mice of multiple spleen or thymus grafting on primary immune responses to sheep red cells. Groups of 18-month-old (AKR × C57Bl)F_1 mice were grafted subcutaneously with twelve 1-day-old C57Bl spleens (twenty-six mice) or twelve 1-day C57Bl thymus glands (twenty-three mice). One month later these grafted mice, along with thirty-two control 2-month-old and 19-month-old (AKR × C57Bl)F_1 mice were immunized with sheep red cells and haemagglutinin responses determined. As may be seen from Fig. 6, haemagglutinin responses in the grafted mice were not significantly elevated above those in control aged mice with the doubtful exception that day 7 titres in grafted mice may have been slightly higher than those in control mice. The mean organ weights in the grafted mice at day 14 after immunization were: *spleen grafted mice* (*spleen grafts* 93±44 mg; *host organs*; spleen 210±60 mg; six subcutaneous lymph nodes 66±12 mg, mesenteric node 72±30 mg; thymus 26±12 mg); *thymus grafted mice* (*thymus grafts* 118±68 mg; *host organs*; spleen 224±54 mg, six subcutaneous lymph nodes 75±30 mg, mesenteric node 98±55 mg; thymus 25±12 mg); *control mice* (spleen 192±58 mg, six subcutaneous lymph nodes 48±15 mg, mesenteric node 79±32 mg, thymus 41±14 mg). In keeping with this lack of change in organ weights, no obvious change in the histology of the spleens in spleen- or thymus-grafted mice was noted from the appearance in the aged control mice.

TABLE 3. Number of plaque-forming cells per spleen in C57Bl mice, 4 days after immunization with sheep red cells

Type of mouse	Age (months)	No. of mice tested	Plaque-forming cells per whole spleen (± Standard Deviation)
Normal	3	8	18545±4600†
Normal	16	13	2660±2500†
Grafted 1–2 months previously with twelve C57Bl spleens	16	9	1625±1600
Grafted 1–2 months previously with twelve C57Bl thymus glands	16	12	4427±2600
Grafted 15 months previously with twelve C57Bl spleens	17	6	3846±3600
Grafted 15 months previously with twelve C57Bl thymus glands	17	5	2938±1650

† Results significantly different ($P<0{\cdot}01$, $t = 10{\cdot}0$). All other results do not differ significantly from those in normal 16-month-old mice.

In additional experiments the effect of splenectomy prior to multiple spleen grafting was investigated. Although splenectomy prior to grafting increased the mean spleen graft weight to 250 mg, no improvement was noted in antibody responses over those in splenectomized mice of this age.

In further experiments, 15-month-old C57Bl mice were grafted subcutaneously with either twelve C57Bl spleens or twelve C57Bl thymus glands. Those grafted mice were not tested until 3 months had elapsed after grafting. However, as in the earlier experiments, haemagglutinin

responses were not significantly higher in grafted mice than in control ungrafted 18-month-old C57Bl mice.

Experiments were carried out also to determine whether multiple spleen or thymus grafting of young mice could increase the already high immune responses of these mice. Groups of twelve 2-month-old C57Bl mice were grafted with twelve spleens or twelve thymus grafts and 2 months after grafting were immunized with sheep red cells, along with 4-month-old control C57Bl mice. No increase in haemagglutinin titres between days 5 and 14 was noted in mice grafted with either spleen or thymus tissue compared with titres in control mice.

Effect of multiple spleen or thymus grafting on haemolytic plaque-forming cell response

Observations were made on groups of 3- and 16-month-old C57Bl mice to determine the total number of plaque-forming cells in the spleen 4 days following immunization with sheep red cells. The results (Table 3) indicated that the plaque-forming cell response was significantly depressed in aged C57Bl mice. Fourteen- to 15-month-old C57Bl mice were grafted subcutaneously with twelve C57Bl 1-day-old spleens or thymus glands and 1–2 months later these mice were tested for their capacity to develop a plaque-forming cell response. The results indicated that the grafted mice showed no improvement of their age-depressed responses, despite the presence of large masses of graft tissue. In separate experiments, an investigation was made to determine whether multiple spleen or thymus grafts made early in life (at the age of 2 months) prevented the age-related decline in immune responses. Such mice were tested at 17 months of age but their 4-day plaque-forming cell response was not elevated above that of control 16-month-old C57Bl mice.

Observations on the spleens of unimmunized aged C57Bl mice indicated that multiple spleen or thymus grafts also failed to raise the background level of plaque-forming cells in the spleen above that in untreated aged mice.

DISCUSSION

The present observations indicate that immune responses to sheep red cells decline with advancing age in some strains of mice but not in others. This system therefore offers a suitable model for an analysis for some of the factors responsible for the age-related decline in immune competence.

The present results confirm earlier observations (Winebright & Fitch, 1962) that the spleen is the major site of production of circulating antibody. Even when immune responses are severely depressed in old age, the spleen apparently continues to be the main site of antibody production. The major morphological change noted to be associated with the depression in immune competence was a decrease in the small lymphocyte content of splenic lymphoid follicles, a disorganization of these follicles and a decrease in active germinal centres in the follicles.

It has been postulated that the thymus is the major site of origin of immunologically competent cells which are then seeded to the spleen and lymph nodes (Burnet, 1962). If this is so then failing thymus function in old age might lead to a slow depopulation of these lymphoid organs and to a decline in immune responses, and certainly thymectomy in adult life accentuates the age-related depression of immune responses (Metcalf, 1965; Miller,

1965; Taylor, 1965). However, the present experiments have shown that the presence of large amounts of neonatal thymus tissue in animals for prolonged periods fails to prevent the onset of age-related immune depression and fails to improve pre-existing depressed immune responses in aged mice. It seems unlikely therefore that the thymus is the major source of antigen-responsive cells or that failing thymus function is the only reason for the depressed immunological responsiveness of old age.

The failure of spleen grafts, which are known to develop the typical morphology of the normal spleen (Metcalf, 1963), to improve immune responses in splenectomized mice was unexpected and remains unexplained. Preliminary investigation of plaque-forming cell responses in such grafts suggests that they contain a normal number of background plaque-forming cells but that these cells do not proliferate at a normal rate following antigenic stimulation. In view of this functional failure of spleen grafts to improve immune responses in young splenectomized animals, no special significance can be attached to the failure of multiple spleen grafts to improve immune responses in aged mice.

Since multiple thymus and multiple spleen grafts did not further increase immune response in young adult animals, it would appear that neither the thymus nor the spleen in the normal young animal is a factor limiting the magnitude of the immune response to sheep red cells.

The exact mechanisms responsible for the decline in competence in the aged animals studied in this investigation remain uncertain. Albright & Makinodan (1966) have reported that the injection of young adult spleen cells into aged mice improved their immune responses, suggesting a shortage of antigen-responsive cells in old age. However, preliminary results in this laboratory have shown that such spleen cell injections are unable to influence immune responses to sheep red cells in aged C57Bl mice and it is possible that different mechanisms are responsible for age-related depressions in immune competence in different strains of mice. In this regard, preliminary studies on the handling of ^{125}I labelled flagellin by the spleen of aged C57Bl mice (Deiner, personal communication) have revealed a failure to concentrate the antigen and a failure to localize the antigen in spleen lymphoid follicles as occurs in the young adult animal. Defective antigen handling, due to disorganization of the splenic lymphoid follicles, therefore may be a factor in determining depressed immune responses in some aged mice.

The severe depression of immune responses in aged (AKR × C57Bl)F_1 mice with reticulum cell sarcoma may be due also to disruption by tumour cells of the architectural arrangement of the spleen lymphoid follicles.

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