Life-Span, Tumor Incidence, and Natural Killer Cell Activity in Mice Selected for High or Low Antibody Responsiveness[1,2,3]

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ABSTRACT—Biozzi mice selected for high (H) or low (L) antibody responsiveness to natural antigens have been followed for their entire life-span to examine their pathology at death. As previously found in selection I, shorter life-span and higher lymphoma incidence were observed in L responder mice than in H responder mice selected for antibody responsiveness to sheep red blood cells (selection II). In mice selected for antibody responsiveness to *Salmonella* flagellar antigens (selection III), similar life-span and similar lymphoma incidence were found in H and L responder mice. Natural killer (NK) cell activity, as assessed in spleen cells from young mice, was lower in L than in H responder mice of selection I but higher in L than in H responder mice of both selections II and III. All these results indicate that longevity and lymphoma incidence at death are independent of NK cell activity in mice selected for H or L antibody responsiveness to natural antigens. Furthermore, genetic selection for antibody responsiveness does not always appear to influence life-span and lymphoma incidence.—JNCI 1984; 72:1127-1136.

Comprehensive studies done by Biozzi et al. (*1*) have demonstrated that immune responsiveness to multideterminant antigens is under polygenic control. By bidirectional selective breeding, H and L responder lines to natural immunogens have been obtained from an independent foundation population of outbred albino mice. Five selections have been done that differ essentially in the antigen and immunization procedure used, the characteristic selected for all selections being the individual maximum and minimum peak antibody responses to an optimal immunization. Thus the characteristic in selection I was the primary antibody response to SRBC and pigeon RBC alternated at each generation, whereas selection II was performed by immunizing all generations only with SRBC, the interval between weaning and immunization being long enough to eliminate maternal antibodies and therefore to avoid their interference with the primary response. Selection III was made for secondary response to the flagellar antigens of two non-cross-reactive *Salmonella* species (*typhimurium* and *oranienburg*), while selection IV was obtained as selection III, but the character selected for was the secondary response to the somatic antigen of *S. typhimurium* and *oranienburg*. Selection V was performed by hyperimmunization with alum-precipitated bovine serum and rabbit gamma globulin and by selection for antibody response. In all selections the H and L responder lines diverged progressively during the consecutive generations of selective breeding until a maximum interline difference in antibody response was reached. At this selection limit, which is attained after a different number of generations in different selections, each line is homozygous at several loci for alleles determining H or L antibody responsiveness to the selection antigen. Thus a variable number of independent loci is involved in each selection (*2*): 9-11 (I), 2-8 (II), 4-7 (III), 2-4 (IV), and 2-4 (V). In all selections, the H or L effects of the alleles at these loci are not limited to the selection antigen but also may influence the immune response to immunogens non-cross-reactive with the selection antigen. This nonspecific effect is not general, since the interline difference in antibody response between H and L responder mice to various unrelated antigens may be identical, smaller, insignificant, or even inverse as compared to the difference in response to the selection antigen. Comparison of the results obtained in the five selections indicates that the nonspecific effect is large in selections I and III, intermediate in selections II and IV, and restricted in selection V (*1*).

Studies done on mice of selection I have indicated that H and L responder mice differ widely in their antibody responses to several antigens but exhibit the same ability to mount T-cell-mediated responses, such as skin graft rejection (*3*), graft-versus-host reaction (*4*), delayed-type hypersensitivity (*5*), and mitotic response to phytohemagglutinin (*6*). In vivo (*7*) and in vitro (*8*) experiments have demonstrated that the genes accumulated in H and L responder lines of selection I are expressed in B-cells but not in helper T-cells. Nonspecific suppressor cells, more numerous or more active in L than in H responder mice, also have been considered to play a role in the regulation of antibody responses in these lines of mice (*9*). The phenotypic expression of genes affecting antibody responsiveness in H and L responder lines of selection I also has been studied at the macrophage level (*8, 10-14*). These

ABBREVIATIONS USED: C=complement; CCM=complete culture medium; E:T=effector-to-target cell; FCS=fetal calf serum; H=high; Ig=immunoglobulin; L=low; MEM=minimum essential medium; NK=natural killer; RBC=red blood cell(s); SRBC=sheep RBC; TL=thymic lymphomas.

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studies have demonstrated that although antigen clearance is the same in H and L responder mice, L responder macrophages are more active than H responder macrophages in antigen uptake and intracellular catabolism, lysosomal enzymatic hydrolysis, and elimination of membrane-bound antigen. This catabolic hyperactivity of L responder macrophages may explain their defective antigen presentation to T-lymphocytes (*14*). The same functional differences between H and L responder macrophages were found in selection II but not in selection III (Biozzi G: Personal communication).

Since phagocytosis and antibody response, as well as cell-mediated immunity, are defense mechanisms against pathogenic microorganisms and neoplastic cells, genetic selection affecting these mechanisms may influence resistance to infections and tumor development. Information from studies performed on mice of selection I suggests that H and L responder mice display different degrees of resistance to pathogens with different aggressive features. A difference in innate resistance to pathogens exists between H and L responder lines, and it can be amplified by vaccination (*15*), but the outcome depends on the pathogen: H responder mice are more resistant than L responder mice against pneumococcus, plasmodia, *Trypanosoma*, and nematode infections (*16–18*) in which antibodies are most effective, whereas L responder mice are more resistant than H responder mice upon infection with intracellular microorganisms such as *Salmonella*, *Yersinia*, *Brucella*, and *Leishmania* (*19–21*), which are inactivated mostly by macrophages. However, the finding that H responder mice are more resistant than L responder mice to infection by virulent *Mycobacterium tuberculosis* (*22*), a well-known intracellular parasite, stresses the limitation of the nonspecific effect brought about by genetic selection. Thus selective breeding accumulates genes that control cell types and functions of the immune system which are relevant to some, but not all, defense mechanisms (*23*). It is, therefore, difficult to predict the impact of each genetic selection on life-span and spontaneous pathology. Whether H and L responder mice of selection I exhibit differences in life-span and pathology has been investigated in a preliminary study done on a few mice in our laboratory. We found a shorter life-span and a higher incidence of spontaneous lymphomas in L responder mice than in H responder mice (*24*). The present study was undertaken to investigate whether also in selection II H responder mice display longer life-span and lower lymphoma incidence. Confirmation of these findings would make it unlikely that during selection for alleles determining H antibody response to SRBC sampling from small populations has randomly drifted L effect alleles at other loci affecting lymphoma incidence, which eventually have become fixed in the H responder line. In addition, selection III was studied to analyze the effect of a different selection antigen on life-span and pathology in H and L responder mice which do not show differences in macrophage functions. Furthermore, NK cell activity in H and L responder mice of selections I, II, and III was assessed to determine whether selective breeding for antibody response also has involved this cell function and whether NK activity is correlated with the incidence of spontaneous lymphomas in H and L responder lines.

MATERIALS AND METHODS

H and L responder Biozzi mice of selections I and II (33d and 25th generations, respectively) were provided by G. Biozzi, Institut Curie, Paris; H and L responder Biozzi mice of selection III (22d generation) were a gift from M. Siqueira, Instituto Biologico, Secretaria da Agricoltura, São Paulo, Brazil. Mice of each H and L responder line were randomly bred in our animal house, and after two generations mice of either sex were randomized and housed 3 to a cage. Mice were given pelleted food and chlorinated water (10–20 ppm free chlorine, pH 2.5) ad libitum. The animal quarters were kept at 20°C and 60% relative humidity. Mice were inspected daily for their entire life-span. In a few cases moribund animals were killed.

Pathology.—Soon after spontaneous death, a complete autopsy was performed on 569 (96%) of the 592 mice under observation. The necropsy included a complete external and internal gross examination. Tissue masses as well as sections of the major organs were taken and processed for histologic examination. Tissues routinely examined were gross lesions, superficial lymph nodes, lungs, thymus, heart, liver, kidneys, stomach, small intestine, ovaries, uterus, spleen, and sternum. The brain was examined grossly, but it was not processed routinely for histopathologic examination. Tissues were fixed in Bouin's fluid and processed for paraffin embedding and sectioning. Sections were stained with hematoxylin and eosin.

The microscopic examination of coded slides was made by the pathologist (V. C.) who recorded his diagnoses on a special form. The collected information was then coded and entered a computer program for statistical analysis. Tumor diagnoses that appeared doubtful were discussed within the Pathology Standardization Committee of the European Late Effect Project Group (*25*).

Statistical analysis of mortality and pathology data.—The occurrence of diseases was evaluated in terms of final incidence, the significance of the differences being tested by corrected χ^2 analysis. Since this procedure may not be informative in all situations, particularly when large differences in mean survival times would bias the comparison among groups or when the small numbers of observed tumors in a group would limit the analysis of latency times, age-related death rates with standard error for specific diseases were computed and plotted as cumulated probabilities as a function of time, according to a model described in detail elsewhere (*26*). In essence, the model used estimates the probability of death for any specified cause (lymphomas or solid tumors) within arbitrarily predetermined nonover-

lapping time intervals of equal length. Within each time interval the number of animals at risk n' was computed according to the relationship $n'=n - 0.5(z + w)$, where n is the number of mice alive at the beginning of the interval, z is the number of mice that died from other causes during the interval, and w is the number of animals withdrawn from the experiment during the interval. In our calculation, w represents the few animals for which no diagnosis was available due to advanced tissue autolysis. The death rate from lymphomas or solid tumors associated with each time interval is then given by the ratio of the number of mice that died from either one specific cause to the number of animals at risk and is therefore corrected for other causes of death and for accidental losses. In summary, the method analyzes both the frequency of lethal diseases and their time of appearance by a single set of statistics that takes competing risks and losses into account and is particularly useful for comparison between H and L responder lines of each selection.

Analysis of NK cell activity.—Spleen cell populations from H and L responder mice of selections I, II, and III were assayed for NK activity. Effector spleen cells were removed from 4- to 10-week-old mice of both sexes and tested for NK activity against 2 tumor cell lines at different E:T ratios. No difference was ever observed in the number of total nucleated spleen cells from H and L responder mice of all three selections. Furthermore, by cell separation and serologic techniques, some phenotypic properties of the effector cells in H and L responder mice of the three selections have been characterized.

Target cells.—Two cell lines were used as targets in cytotoxicity assays: YAC-1, derived from a Moloney virus-induced lymphoma developed in A/Sn mice, and JURKAT, a murine NK cell-susceptible human cell line. Cell lines were maintained in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) supplemented with gentamicin (10 μg/ml), 10% heat-inactivated FCS (GIBCO), 4 m*M* L-glutamine, and 1% sodium pyruvate.

Effector cells.—For preparation of spleen cell suspensions, spleens from 3 to 5 mice were forced through a stainless steel mesh. Spleen cells were depleted of RBC by hypotonic shock. Cell suspensions were washed once in RPMI-1640 supplemented with 10% FCS (CCM) and resuspended in 0.1 × MEM (GIBCO). Fifteen seconds later an equal volume of double-strength MEM was added. Thereafter, spleen cells lacking RBC were washed twice in CCM, passed through a 100-μm nylon filter to remove debris, counted, and suspended at the appropriate cell concentration.

Plastic adherence.—After RBC lysis, 6×10^7 effector spleen cells were suspended in 6 ml CCM and incubated for 2 hours at 37°C in 100-mm petri dishes (A/S Nunc, Copenhagen, Denmark) in a humidified atmosphere of 10% CO_2 in air. At the end of the incubation period, nonadherent cells were removed by vigorous shaking, and the dishes were thoroughly washed three times with warm CCM. Adherent cells, to be used as control, were then recovered by the plates being scraped with a rubber policeman; the cells were then washed, counted, and resuspended in CCM. Phagocytic cells in the adherent population ranged from 85 to 90% as judged by latex bead injection.

Treatment with anti-asialo GM_1 antiserum.—Five milliliters of effector cell suspension (10^7 cells/ml) was incubated with 0.1 ml anti-asialo GM_1 antiserum for 45 minutes at 4°C. The cells were then washed twice with RPMI-1640 without FCS, resuspended in guinea pig C (Sclavo, Siena, Italy) preabsorbed with agarose (80 mg/ml) and spleen cells (1 spleen/ml), at 1:10 final dilution, incubated for 45 minutes at 37°C, and then washed three times. The anti-asialo GM_1 antiserum was a gift from K. Okumura, Department of Immunology, University of Tokyo.

Enrichment of Ig^- cells.—Ig^- cell populations were obtained as described by Mage et al. (*27*). Briefly, 5×10^7 effector spleen cells, after RBC lysis by hypotonic shock, were incubated for 1 hour at 4°C in 100-mm petri dishes that had been coated overnight with 5 ml affinity-purified goat anti-mouse Ig antibodies (100 μg/ml). Nonadherent cells, recovered by gentle swirling, were usually 30% of the input cells. This procedure routinely yielded cell populations containing less than 5% Ig^+. Adherent cells (Ig^+ cells) were recovered by the plates being scraped with a rubber policeman; the cells were then washed twice and used as control.

NK cell activity assay.—The NK cytotoxicity assay was performed with the use of 2 tumor cell lines, YAC-1 and JURKAT, as target cells. Briefly, 10^7 target cells in 0.1 ml CCM were mixed with 0.1 mCi $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Amersham, England) and incubated for 1 hour at 37°C. Then the cells were washed three times in CCM and seeded (10^4 cells/well) in 96-well round-bottom microplates (A/S Nunc). Thereafter, each well received 0.1 ml CCM containing $1.25\text{-}10\times10^5$ effector spleen cells. After 4 hours of incubation at 37°C, the plates were centrifuged at 200×*g* for 10 minutes, and the radioactivity in 0.1 ml supernatant was measured in a gamma counter. Spontaneous release of ^{51}Cr radioactivity was determined in cultures in which effector spleen cells were replaced by unlabeled target cells. Spontaneous release was routinely 5–10% of the maximum release obtained from Nonidet P40-treated labeled target cells. The percent cytotoxicity was calculated from the radioactivity (cpm) released in experimental and control cultures as follows: Percent cytotoxicity=[(experimental release − spontaneous release)/(maximum release − spontaneous release)]×100. The percent values are presented as the mean of triplicate determinations.

RESULTS

Longevity and Pathology of Mice of Selection II

The mean life-span of H and L responder mice, separately determined for males and females, is shown in table 1. The life-span is much shorter in L responder mice than in H responder mice of both sexes, a

maximum difference of approximately 300 days being observed between males of the 2 lines. Also, the difference between females is statistically significant ($P<.001$). Curves of cumulative mortality from all causes are shown in text-figure 1 for male and female mice. In both sexes the curves indicate a faster initial rise of mortality in L responder mice followed by a parallel increase of cumulative mortality in H and L responder mice. Thus the difference in the initial rise accounts for the shorter mean life-span observed in L responder mice.

Table 1 also shows the distribution of lymphoid neoplasms and solid tumors in H and L responder mice of both sexes. Nonthymic malignant lymphoma is the only type of lymphoid neoplasms seen in H responder mice. The tumor is a lymphohistiocytic lymphoma with a nodular and diffuse distribution. It is constantly associated with gross involvement of the spleen, liver, and mesenteric lymph node. In some cases other deep and superficial lymph nodes and Peyer's patches also are involved. Morphologically, the tumor is predominantly composed of large, undifferentiated, moderately pleomorphic cells with cleaved nuclei. TL, absent in H responder mice, develop in 30% of L responder mice, mostly in females. The sex difference in L responder mice is statistically significant ($.01<P<.05$). Animals dying from TL show at necropsy a large, white, soft mass that occupies the mediastinum. The lungs are often atelectatic. Invasion of the spleen and lymph nodes may occur when the tumor becomes generalized. Morphologically, the thymus is entirely replaced by highly immature lymphocytes, uniform in size, with scanty cytoplasm and abundant nuclear chromatin. Benign and malignant solid tumors of several types also are observed, the most frequent types being localized in lungs, liver, and skin. Tumors of other sites are less frequent and irregularly distributed among groups. The incidence of solid tumors is higher ($P<.001$) in females than in males of the H responder line, whereas it is similar in the two sexes of the L responder line. The incidence of solid tumors is higher in H responder mice than in L responder mice of both sexes, but the difference is statistically significant ($P<.001$) only in females.

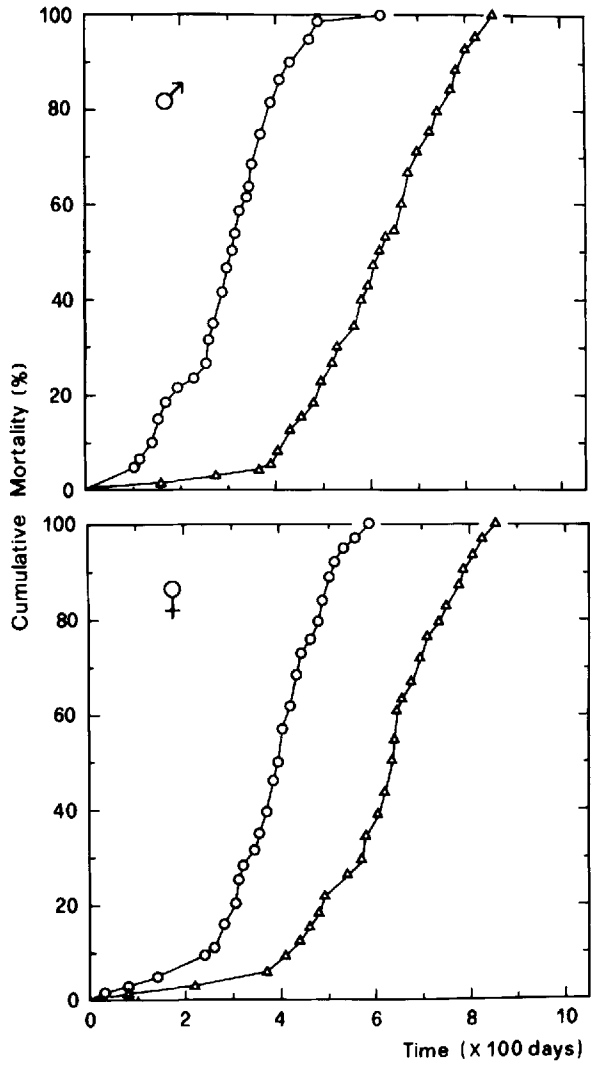
Lymphoid neoplasms are more frequent in L responder mice than in H responder mice of both sexes, but the difference is statistically significant ($P<.001$) only in females. Comparison of the final incidence of solid tumors versus all lymphoid neoplasms indicates that solid tumors are more frequent in H responder mice of both sexes, whereas lymphomas are more frequent in L responder mice of both sexes.

The data in table 1 suggest that lymphoid neoplasms are the major cause of life-span shortening in L responder mice of both sexes. At variance, solid tumors seem to have no influence on life-span shortening since their incidence is higher in H than in L responder mice of both sexes. The analysis of age-related death rates for lymphomas or solid tumors in H and L responder mice of both sexes has been crucial to correlate life-span and specific cause of death. As reported in text-figure 2, the onset of lymphomas occurs earlier and their development is faster in L responder mice than in H responder mice of both sexes,

TABLE 1.—*Life-span and neoplasms in mice of selection II*

Parameter	H responder mice		L responder mice	
	Males	Females	Males	Females
No. of mice per group	70	64	60	63
Mean life-span, days ± SD	613±145	611±153	305±110	386±111
No. of autopsied mice	67	64	58	61
Lymphoid neoplasms				
Malignant lymphoma	9	8	6	19
TL			4	14
Total (%)	9 (13)	8 (12)	10 (17)	33 (54)
Solid tumors: site and tumor type				
Lung				
Alveolar adenoma	3	6		
Alveolar adenocarcinoma	2	4		
Liver, hepatocellular adenoma	3	1	4	
GI[a] tract, adenocarcinoma		1		
Adrenal, cortical adenoma	1	1		
Bladder, carcinoma			1	
Skin				
Squamous cell carcinoma	2	5		5
Fibrosarcoma		1		1
Rhabdomyosarcoma	1	2		
Bone, osteogenic sarcoma		3		
Vascular system, hemangioendothelioma		3	1	
Mammary gland, adenocarcinoma		2		3
Ovary, tubular adenoma		1		
Total (%)	12 (18)	30 (47)	6 (10)	9 (15)

[a] Gastrointestinal.



TEXT-FIGURE 1.—Cumulative mortality curves for H (△) and L (○) responder male and female mice of selection II.

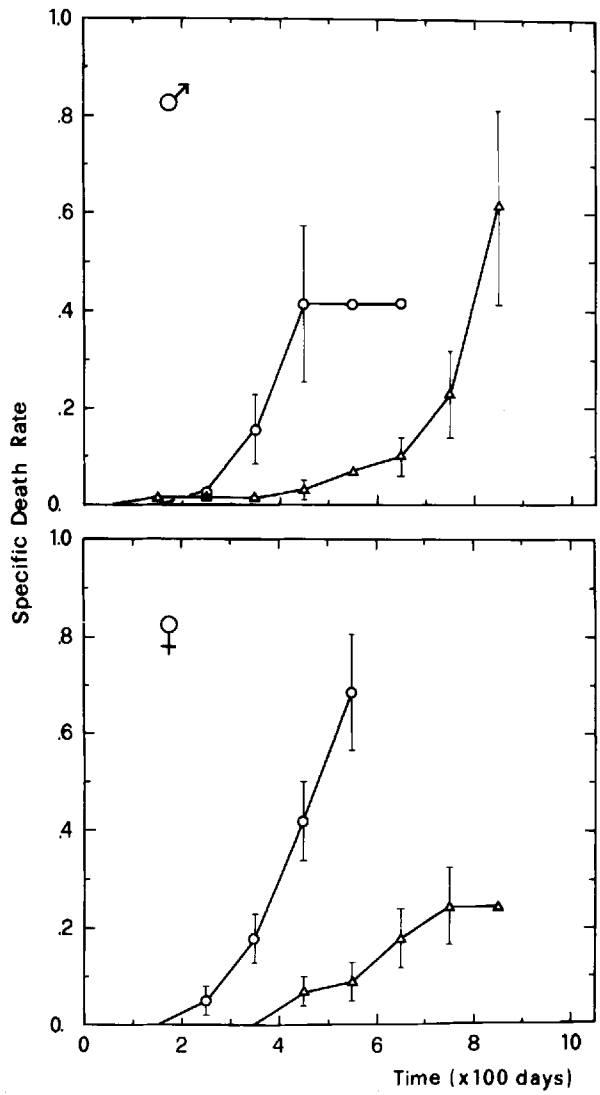
mostly in females. In contrast to the final incidence of solid tumors, which is higher in H than in L responder females (table 1), the curves of text-figure 3 indicate that the death rate from these tumors is accelerated in both sexes during the life-span of L responder mice. Thus, although H responder mice eventually exhibit a higher percentage of solid tumors, L responder mice have a reduced life-span and are at higher risk of dying also from these tumors.

Inflammatory diseases, mostly pneumonia, were found randomly distributed among all groups, Glomerulosclerosis, frequently associated with poliarteritis, was the major degenerative lesion observed. These nonneoplastic diseases were equally frequent in males and females of the H and L responder lines of selection II.

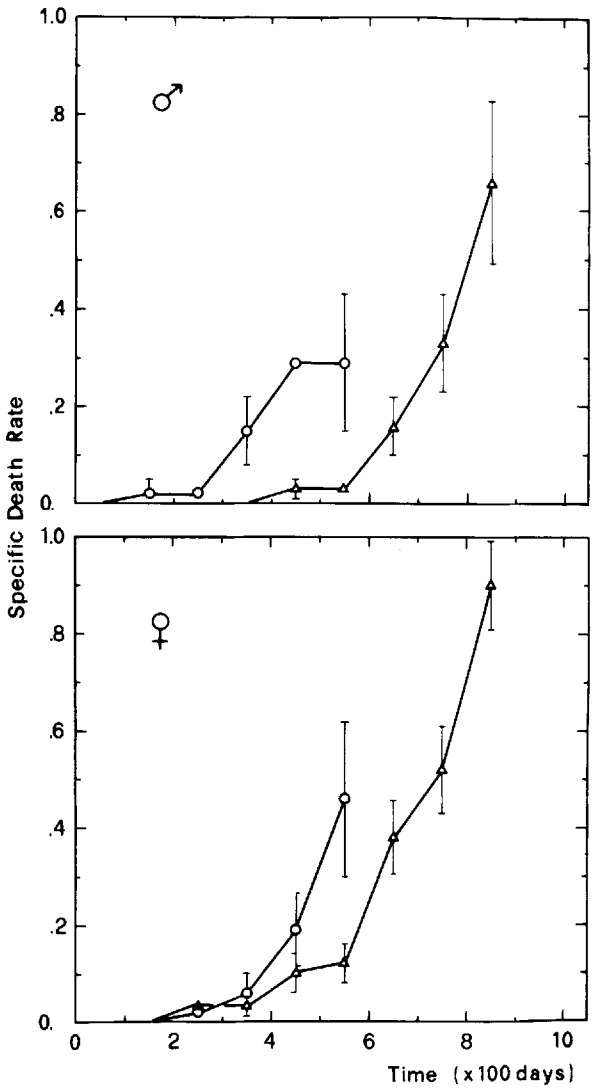
Longevity and Pathology of Mice of Selection III

As shown in table 2, no difference was observed in mean life-span between H and L responder mice of selection III. This is also apparent from the overlapping cumulative mortality curves for H and L responder mice of either sex (text-fig. 4).

Lymphoma incidence is not statistically different in H and L responder mice of either sex (table 2). However, the percentage of these cancers is lower ($.01<P<.05$) in males than in females of the L responder line. Most of the solid tumors are localized in lungs and liver. Neoplasms in other sites have been seen rarely in these mice, with the exception of subcutaneous fibrosarcomas found with high frequency in females of both H and L responder lines. This finding is in line with previous observations in females of different mouse strains (*28, 29*). The percentage of solid tumors is the same in H and L responder mice of either sex, and the final incidence of these tumors is greater than that of lymphomas in both lines, as observed in



TEXT-FIGURE 2.—Cumulative death rate specific for lymphoid neoplasms in H (△) and L (○) responder male and female mice of selection II as a function of time (consecutive nonoverlapping periods of 100 days). The rate is corrected for other causes of death and for accidental losses.



TEXT-FIGURE 3.—Cumulative death rate specific for solid tumors in H (△) and L (○) responder male and female mice of selection II as a function of time. *See* text-fig. 2.

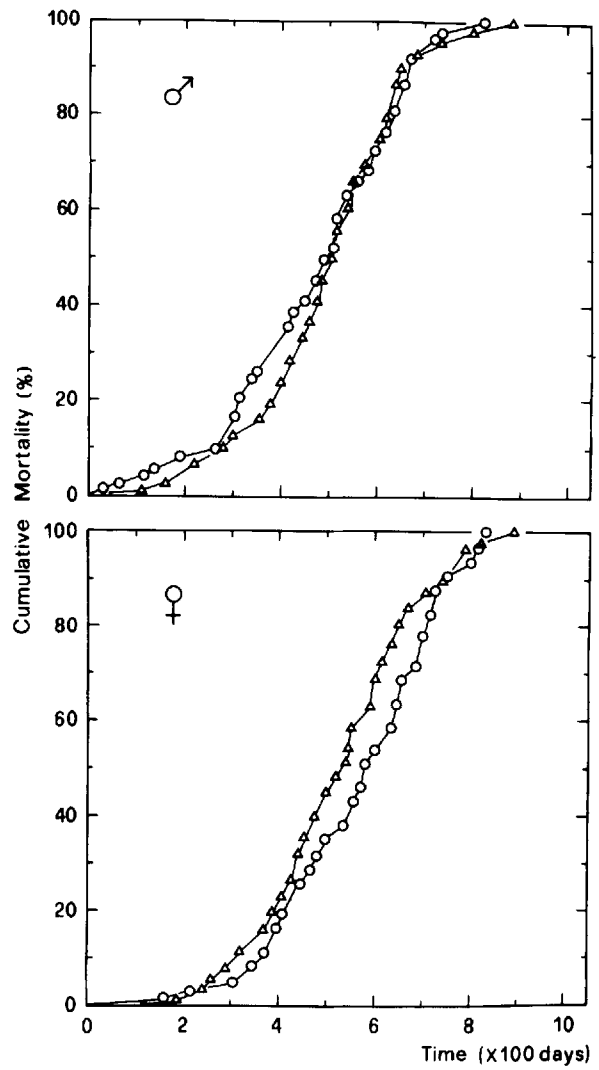
the H responder line of selection II. Analysis of age-specific death rates for lymphomas or solid tumors in males and females of selection III revealed complete overlapping of the cumulative mortality curves for H and L responder mice (data not shown). Altogether, these findings indicate that in selection III the life-span as well as the incidence and rate of all neoplasms are the same in H and L responder mice.

Inflammatory and degenerative diseases also were found in selection III, but, as in selection II, their types and frequencies were randomly distributed between H and L responder mice of both sexes.

NK Activity of Spleen Cells From Mice of Selections I, II, and III

As shown in text-figure 5, NK cell activity against YAC-1 (*upper panel*) or JURKAT (*lower panel*) target cells is higher in H responder mice than in L responder mice of selection I. The reverse was found for selections II and III inasmuch as L responder mice displayed higher NK cell activity than did H responder mice. The NK activity is almost identical for L responder mice of all three selections, whereas the NK activity of H responder mice from selection I is particularly high and contrasts with the lower NK activity of H responder mice from selections II and III. No difference between sexes was ever noticed in each selection. These results were obtained with pooled spleen cells from 3–5 mice; similar findings were observed with spleen cells from individual mice (table 3).

Control experiments were done on spleen cells from mice of all three selections to demonstrate that cytotoxicity was mediated by NK cells (data not shown). Since NK cells express the membrane antigen asialo GM_1 (*30*), spleen cells from H and L responder mice of each selection were incubated with anti-asialo GM_1 antiserum and C before the assay for NK cell activity. The cytotoxic treatment with anti-asialo GM_1 anti-



TEXT-FIGURE 4.—Cumulative mortality curves for H (△) and L (○) responder male and female mice of selection III.

TABLE 2.—*Life-span and neoplasms in mice of selection III*

Parameter	H responder mice		L responder mice	
	Males	Females	Males	Females
No. of mice per group	112	87	73	63
Mean life-span, days ± SD	496±150	527±156	474±175	570±156
No. of autopsied mice	107	82	68	62
Lymphoid neoplasms				
Malignant lymphoma	13	9	2	11
TL		1	1	1
Total (%)	13 (12)	10 (12)	3 (4)	12 (19)
Solid tumors: site and tumor type				
Lung				
Alveolar adenoma	13	7	2	
Alveolar adenocarcinoma	9	3	8	4
Liver, hepatocellular adenoma	4	4		
GI[a] tract, adenocarcinoma		1		
Adrenal, cortical adenoma	1	2		1
Skin				
Squamous cell carcinoma	1	1		
Fibrosarcoma	2	13	4	11
Rhabdomyosarcoma				3
Vascular system, hemangioendothelioma		2		
Mammary gland				
Adenoma		2		1
Adenocarcinoma				1
Ovary, tubular adenoma		1		8
Total (%)	30 (28)	36 (44)	14 (21)	29 (47)

[a] Gastrointestinal.

bodies and C completely abrogates NK activity of spleen cells from H and L responder mice of each selection. However, since the asialo GM_1 antigen is also shared by a macrophage subset (*31*), macrophages were removed from the spleen cell population by plastic adherence to test whether these cells could contribute to cytotoxicity. It was found that NK activity of spleen cells from H and L responder mice of selections I, II, and III is not reduced after macrophage removal. Actually, cytotoxicity of the nonadherent cell population is increased as a consequence of NK cell enrichment. Conversely, adherent macrophages recovered from plastic plates are in all selections devoid of NK activity, although they are able to ingest latex beads. Finally, the contribution of B-lymphocytes to NK activity was ruled out by cell separation on anti-Ig antibody-coated plates. After elimination of B-lymphocytes (adherent Ig^+ cells), NK activity is either unchanged or increased as compared to unseparated spleen cells in all selections, while Ig^+ cells recovered from the antibody-coated plates exhibit negligible cytotoxicity.

In conclusion, the observed differences in cell cytotoxicity between H and L responder mice of selections I, II, and III can be attributed to NK cells rather than to macrophages or B-lymphocytes, cells in which selected genes are known to be phenotypically expressed (*1*).

DISCUSSION

The patterns of mortality and pathology observed in mice of selection II confirm and extend our previous finding (*24*) of shorter life-span and higher incidence of lymphomas in L responder mice than in H responder mice of selection I. Since the primary antibody response to SRBC is a characteristic selected for in both selections, the consistency of the mortality and pathology data from both studies makes it unlikely that during selection for antibody responsiveness alleles at other loci, which affect life-span and lymphoma incidence, have been randomly drifted by sampling from small populations and have subsequently become fixed. Conversely, the data favor the possibility that high-effect alleles for antibody responsiveness either have pleotropic positive effects on life-span and negative effects on lymphoma incidence or are preferentially associated (linkage disequilibrium) with alleles at other loci with high effects on life-span and low effects on lymphoma incidence.

The lack of any association between antibody response to *Salmonella* flagellar antigen and both life-span and lymphoma incidence in H and L responder mice of selection III indicates that the genes selected for this antibody response have no pleotropic effects nor are they linked to other genes acting on the other two traits. Moreover, random drift appears irrelevant to the results of selection III.

The difference in mean life-span (table 1) and cumulative mortality (text-fig. 1) between H and L responder mice of selection II is very large. The life-span of L responder mice is significantly lower than that of the majority of mouse strains examined (*26, 32-35*). Since inflammatory and degenerative disease were randomly distributed among mice of selections II and III (data

TABLE 3.—*NK activity of spleen cells from individual mice*[a]

Selection	Expt No.	Responder line	Sex	Age, days	Percent cytotoxicity at E:T ratio of:[b] 25	50	100
I	1	H	♂	73	11.9	15.1	21.2
		H	♂		17.3	20.5	21.9
		H	♀		10.2	14.7	21.8
		H	♀		11.3	15.1	19.1
					12.7±1.6	*16.3±1.4*	*21.0±0.6*
		L	♂	63	2.5	4.5	6.0
		L	♂		2.7	3.9	6.3
		L	♀		2.0	3.1	7.3
		L	♀		3.5	4.8	8.0
					2.7±0.3	*4.0±0.4*	*6.9±0.5*
	2	H	♂	35	17.5	26.9	30.1
		H	♂		19.0	26.3	27.1
		H	♀		23.5	37.8	36.9
		H	♀		24.2	36.7	49.5
					21.0±1.6	*31.9±3.0*	*35.9±5.0*
		L	♂	48	16.5	23.2	18.0
		L	♂		8.9	11.8	17.8
		L	♀		17.3	30.7	34.8
		L	♀		7.6	9.4	9.7
					12.6±2.5	*18.8±4.9*	*20.0±5.2*
II	1	H	♂	32	5.9	10.7	16.0
		H	♂		7.6	10.2	14.8
					6.7±0.8	*10.4±0.2*	*15.4±0.6*
		L	♂	32	12.5	16.5	25.6
		L	♂		13.7	14.1	19.0
					13.1±0.6	*15.3±1.2*	*22.3±3.3*
	2	H	♂	30	17.0	8.2	28.2
		H	♂		15.0	20.0	39.0
					16.0±1.0	*14.1±5.9*	*33.6±5.4*
		L	♂	28	18.0	26.4	43.0
		L	♂		29.2	37.3	61.6
					23.6±5.6	*31.9±5.4*	*52.3±9.3*
III	1	H	♂	35	3.7	4.1	7.2
		H	♂		3.2	3.6	5.3
		H	♂		4.0	4.6	7.9
					3.6±0.2	*4.1±0.3*	*6.8±0.8*
		L	♂	36	15.0	17.4	22.0
		L	♂		13.0	16.2	20.3
		L	♂		13.0	15.2	21.1
					13.7±0.7	*16.3±0.6*	*21.1±0.5*

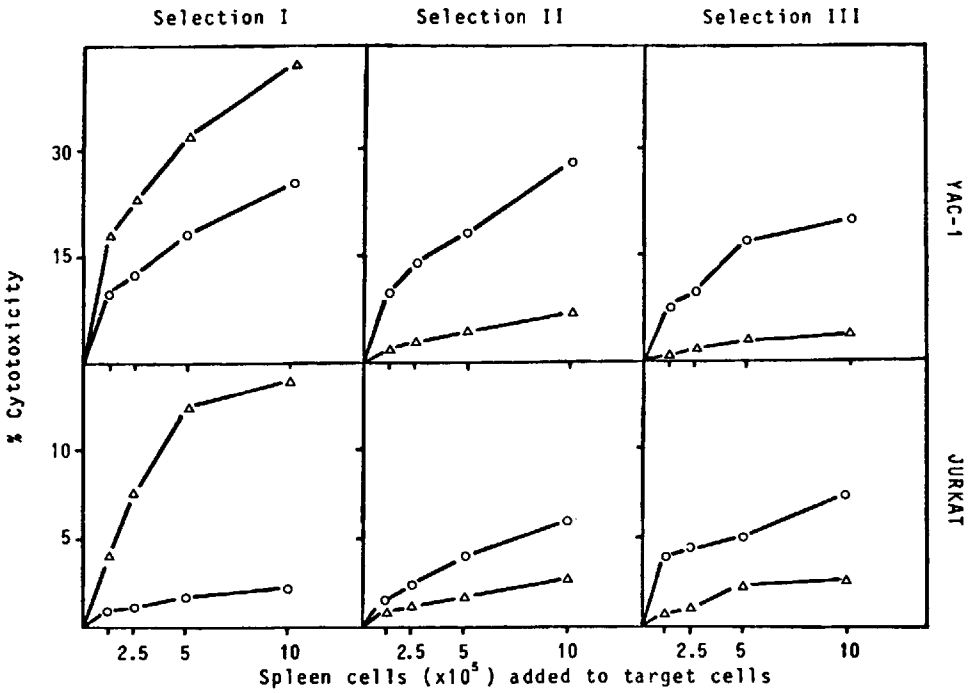
[a] Maximum release and spontaneous release of ^{51}Cr (cpm) were as follows: In selection I: expt 1, 1,016 and 78; expt 2, 513 and 60. In selection II: expt 1, 570 and 66; expt 2, 447 and 150. In selection III: expt 1, 892 and 65.

[b] *Numbers in italics* are means ± SE.

not shown), the difference in life-span between L and H responder mice of selection II appears to reflect the different rate of appearance of lymphomas (text-fig. 2) and solid tumors (text-fig. 3). Indeed, both cancers exhibit earlier onset and faster development in L responder mice than in H responder mice of selection II but no differences in L and H responder mice of selection III, which display the same mean life-span and cumulative mortality. Comparison of data in text-figures 2 and 3 clearly indicates that in selection II the death rate in L responder mice of both sexes, but mostly in females, is higher for lymphomas than for solid tumors.

The different incidence of lymphomas in H and L responder mice of selection I (*24*) or II might suggest a causal relationship between antibody responsiveness and antitumor immunity. This possibility, however, is contradicted by the same incidence of lymphomas in H and L responder mice of selection III. The nonspecific effects of the three selections were measured from the responses to different antigens, but they were found comparable in terms of amplitude of the interline difference. The nonspecific effect is very broad in selections I and III and somewhat intermediate in selection II (*1*). Thus H and L responder mice of selections I, II, and III exhibit a similar difference in antibody responsiveness to a large variety of antigens, yet lymphoma incidence is quite dissimilar in selections I and II as compared to that in selection III. It appears, therefore, that H antibody responsiveness is not always associated with resistance to spontaneous lymphomas. This difficulty in correlating antibody responsiveness and antitumor immunity also was met in previous studies on nonspontaneous tumors (*1*). It was found that transplantable syngeneic leukemias, mammary carcinoma, lymphosarcoma, and allogeneic Ehrlich carcinoma grow equally well in recipients selected for H or L antibody response to SRBC, whereas allogeneic sarcoma 180 grows even faster in H responder mice than in L responder mice. So far, only tumors induced by benzo[*a*]pyrene develop with higher frequency in the L responder mice than in H responder mice of selections I and II. The incidence of carcinogen-induced tumors and of spontaneous lymphomas in mice of selections I and II suggests that L responder macrophages, which are very active in enzymatic hydrolysis and intracellular catabolism, do not play any evident role in counteracting the development of these neoplasms. Thus the antitumor immunity of H and L responder mice cannot be associated readily with the catabolic function of macrophages or with the antibody responsiveness of B-lymphocytes nor can it be related to T-cell functions that have been shown repeatedly to be similar in H and L responder mice.

NK activity of spleen cells, which several studies (*36*) indicate as a major defense mechanism against tumor growth, was thoroughly investigated in H and L responder mice of selections I, II, and III. Contributions of macrophages and B-lymphocytes to NK activity in the assay used were ruled out for the three selections. As shown in text-figure 5, NK cell activity against YAC-1 and JURKAT tumor target cells is higher in H than in L responder mice of selection I, but it is lower in H than in L responder mice of both selections II and III. The opposite results obtained in selections I and II indicate that genes for NK cell activity or for susceptibility to neoplastic transformation may be randomly drifted in association with genes selected for H or L antibody response to SRBC. Conversely, the



TEXT-FIGURE 5.—NK activity of spleen cells from H (Δ) and L (O) responder mice of selections I, II, and III. *Upper panel:* Target, 10^4 YAC-1 lymphoma cells. *Lower panel:* Target, 10^4 JURKAT lymphoma cells. Maximum release and spontaneous release of ^{51}Cr (cpm) were as follows: in selection I, 250 and 60 for YAC-1 and 1,996 and 139 for JURKAT; in selection II, 1,456 and 103 for YAC-1 and 1,996 and 139 for JURKAT; in selection III, 1,624 and 102 for YAC-1 and 3,045 and 206 for JURKAT.

interline difference in favor of L responder mice in selection III suggests that genes selected for H antibody response to *Salmonella* flagellar antigen exhibit pleotropic negative effects or preferential linkage with L effect genes acting on NK cell activity, whereas genes involved in tumor induction apparently are not affected by this selection. However, random drift of genes for NK cell activity cannot be excluded.

Comparison of lymphoma incidence and NK cell activity in selections I, II, and III indicates that NK cell activity does not play a major role in defense mechanisms against spontaneous lymphomas in these mice. As illustrated in table 4, the expected correlation is found only in selection I in which L responder mice exhibit lower NK activity and higher lymphoma incidence as compared to those in H responder mice. In selections II and III, NK activity is higher in L responder mice than in H responder mice, but lymphoma incidence is similar or even higher in L responder mice as compared to that in H responder mice. Thus NK activity appears to be unrelated to the development of spontaneous lymphomas in these mice. However, NK activity was determined on spleen cells from 4- to 10-week-old mice while lymphomas appeared much later (text-fig. 2). Possibly, during the latent period NK cells undergo changes in number and/or activity which may not reflect that found in younger animals and may be effective in antitumor immunity. Evaluation of NK activity in pyran-stimulated aged H and L responder mice is under way in our laboratory. Also, correlation studies on NK activity and incidence of benzo[*a*]pyrene-induced tumors in young H and L responder mice should contribute relevant information to this controversial issue.

TABLE 4.—*Life-span, lymphoma incidence, and NK activity in H and L responder mice*

Selection	Life-span	Lymphoma incidence	NK activity
I	L < H	L > H	L < H
II	L < H	L > H	L > H
III	L = H	L = H	L > H

In conclusion, results from selection II, which confirm and extend our previous observations on mice from selection I (*24*), together with results from selection III, indicate that genetic selection for antibody responsiveness to some but not other natural antigens may affect longevity and lymphoma incidence. Thus in selection II L responder mice exhibited shorter lifespans and higher lymphoma incidence as compared to H responder mice, but no difference was observed between L and H responder mice of selection III. When NK cell activity was assessed in spleen cells from young mice, it was found to be lower in L than in H responder mice of selection I but higher in L than in H responder mice of both selections II and III. These findings indicate that longevity and lymphoma incidence at death are independent from NK cell activity in mice selected for H or L antibody responsiveness to natural antigens.

REFERENCES

(*1*) BIOZZI G, MOUTON D, SANT'ANNA OA, et al. Genetics of immunoresponsiveness to natural antigen in the mouse. Curr Top Microbiol Immunol 1979; 85:31-98.

(2) Biozzi G, Siqueira M, Stiffel C, Ibanez OM, Mouton D, Ferreira VC. Genetic selections for relevant immunological functions. In: Fougereau M, Dausset Y, eds. Immunology 80. New York: Academic Press, 1980:432-457.

(3) Liacopulos-Briot M, Bouthillier Y, Mouton D, et al. Comparison of skin allograft rejection and cytotoxic antibody production in two lines of mice genetically selected for "high" and "low" antibody synthesis. Transplantation 1972; 14: 590-596.

(4) Byfield PE, Howard JG. Equivalent graft versus host reactivity of spleen cells from two lines of mice genetically selected for high and low humoral antibody formation. Transplantation 1972; 14:133-134.

(5) Mouton D, Bouthillier Y, Oriol R, Decreusefond C, Stiffel C, Biozzi G. Intensité de la réaction d'hypersensibilité retardée chez les souris des lignes sélectionnées "bonnes" et "mauvaises" productrices d'anticorps. Ann Immunol (Institut Pasteur) 1974; 125C:581-588.

(6) Liacopulos-Briot M, Mouton D, Stiffel C, et al. Réponse a la phytohemagglutinine des lymphocytes de 2 lignes de souris génétiquement sélectionnées pour une forte et une faible synthèse d'anticorps. Ann Immunol (Institut Pasteur) 1974; 125C:393-404.

(7) Howard JG, Christie GH, Courtenay BM, Biozzi G. Studies on immunological paralysis. VIII. Pneumococcal polysaccharide tolerance and immunity differences between the Biozzi high and low responder lines of mice. Eur J Immunol 1972; 2:269-273.

(8) Doria G, Agarossi G, Biozzi G. In vitro immune response of spleen cells from mice genetically selected for high or low antibody production. Immunology 1978; 34:999-1005.

(9) Nakano K, Cinader B, Sabiston BH. Age-dependent changes in tolerizability with rabbit gamma-globulin in the Biozzi high and low responder lines of mice. Eur J Immunol 1979; 9:1014-1017.

(10) Biozzi G, Stiffel C, Mouton D, Bouthillier Y, Decreusefond C. La régulation génétique de la sinthèse des immunoglobulines au cours de la réponse immunologique. Ann Immunol (Institut Pasteur) 1974; 125C:107-142.

(11) Wiener E, Bandieri A. Differences in antigen handling by peritoneal macrophages from the Biozzi high and low responder lines of mice. Eur J Immunol 1974; 4:457-463.

(12) Howard JG, Courtenay BM, Desaymard C. Equivalent responsiveness to branched polysaccharides and their dinitrophenyl conjugates in the Biozzi high and low responder lines of mice. Eur J Immunol 1974; 4:453-457.

(13) Ishizaka S, Otani S, Morisawa S. Effects of carrageenan on immune responses. II. A possible regulatory role of macrophages in the immune responses of low responder mice. J Immunol 1978; 120:61-65.

(14) Adorini L, Doria G. Defective antigen presentation by macrophages from mice genetically selected for low antibody response. Eur J Immunol 1981; 11:984-989.

(15) Biozzi G, Mouton D, Stiffel C, Sant'Anna OA, Bouthillier Y. The genetic regulation of antibody responsiveness to natural immunogens in relation to the protective effect of vaccination. In: Werner GH, Floc'h F, eds. Pharmacology of immunoregulation. New York: Academic Press, 1978:123-139.

(16) Heumann AM, Stiffel C, Monjour L, Bucci A, Biozzi G. Correlation between genetic regulation of antibody responsiveness and protective immunity induced by Plasmodium Berghei vaccination. Infect Immun 1979; 24:829-836.

(17) Kierszenbaum F, Howard JG. Mechanism of resistance against experimental trypanosoma Cruzi infection: The importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. J Immunol 1976; 116:1208- 1211.

(18) Jenkins DC, Carrington TS. Hematospiroides dubius: The course of primary, secondary and tertiary infections in high and low responder Biozzi mice. Parasitology 1981; 82:311-318.

(19) Biozzi G. Differential susceptibility of high and low antibody producing mouse lines to infection and tumor transplantation. In: McDevitt HO, Landy M, eds. Genetic control of immunoresponsiveness. New York: Academic Press, 1972: 317-322.

(20) Cannat A, Bousquet C, Serre A. Response of high and low antibody producers to *Brucella*. Ann Immunol (Institut Pasteur) 1978; 129C:669-683.

(21) Hale C, Howard JG. Immunological regulation of experimental cutaneous Leishmaniasis. 2. Studies with Biozzi high and low responder lines of mice. Parasite Immunol 1981; 3:45-56.

(22) Lagrange PH, Hurtrel B, Thichstun PM. Immunological behavior after mycobacterial infection in selected lines of mice with high or low antibody responses. Infect Immun 1979; 25:39-47.

(23) Biozzi G, Cabrera WH, Mouton D, Ibanez OM. Restricted and general polygenic regulation of antibody responsiveness. In: Benacerraf B, ed. Immunogenetics and immune regulation. Lecture Schiapparelli. Milan: Masson Italia Editori, 1982; 31-60.

(24) Covelli V, Di Majo V, Bassani B, Metalli P. Life-span and spontaneous tumours in mice with high and low antibody responses (Biozzi mice). Br J Cancer 1978; 38:472-475.

(25) EULEP Pathology Atlas (European Late Effects Project Group, Comittee on Pathology Standardization). Neuherberg: Gesellschaft für Strahlen und Unweltforschung MBH, 1980.

(26) Covelli V, Di Majo V, Bassani B, Silini G, Metalli P. Role of the spleen in spontaneous reticulum cell sarcoma of (C57BL/Cne × C3H/Cne)F_1 mice. JNCI 1980; 64:139-144.

(27) Mage MG, McHugh LL, Rothstein TS. Mouse lymphocytes with and without surface immunoglobulins: Preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. J Immunol Methods 1977; 15:47-53.

(28) Dunn TB, Heston WE, Deringer MK. Subcutaneous fibrosarcomas in strain C3H and C57BL female mice and F_1 backcross hybrids of these strains. J Natl Cancer Inst 1956; 17:639-655.

(29) Covelli V, Di Majo V, Bassani B, Metalli P, Silini G. Radiation-induced tumors in transplanted ovaries. Radiat Res 1982; 90:173-186.

(30) Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T. A glycolipid on the surface of mouse natural killer cells. Eur J Immunol 1980; 10:175-180.

(31) Okumura K, Habu S. The role of asialo GM_1^+ (GA_1^+) cells in the resistance to transplants of bone marrow or other tissues. In: Herberman RB, ed. NK cells and other natural effector cells. New York: Academic Press, 1982:1527-1533.

(32) Holland JM, Mitchell TJ, Gipson LC, Whitaker MS. Survival and cause of death in aging germfree athymic nude and normal inbred C3H/He mice. JNCI 1978; 61:1357-1361.

(33) Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F_1 hybrids. J Natl Cancer Inst 1973; 50:1195-1213.

(34) Stutman O. Immunodepression and malignancy. Adv Cancer Res 1975; 22:261-422.

(35) Ward JM, Goodman DG, Squire RA, Chu KC, Linhart MS. Neoplastic and nonneoplastic lesion in aging (C57BL/6N × C3H/HeN)F_1 (B6C3F_1) mice. JNCI 1979; 63:849-854.

(36) Herberman RB, ed. NK cells and other natural effector cells. New York: Academic Press, 1982:1047-1166.