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

Vincenzo Covelli,[4,5] Stefano Marini,[4] Vincenzo Di Majo,[4] Bruno Bassani,[4] Camillo Mancini,[4] Luciano Adorini,[4] *and* Gino Doria[4]

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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ntigen uptake and intra-
nal enzymatic hydrolysis,
ane-bound antigen. This
responder macrophages
antigen presentation to
me functional differences
macrophages were found
selection III (Biozzi G:

tibody response, as well as
efense mechanisms against
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nder mice display different
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innate resistance to patho-
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n (*15*), but the outcome
responder mice are more
mice against pneumococ-
na, and nematode infec-
bodies are most effective,
re more resistant than H
n with intracellular micro-
a, *Yersinia*, *Brucella*, and
are inactivated mostly by
finding that H responder
in L responder mice to
cterium tuberculosis (*22*),
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on II H responder mice
lower lymphoma inci-
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the H responder line. In
died to analyze the effect

cell function and whether NK activity is correlated w
the incidence of spontaneous lymphomas in H and
responder lines.

MATERIALS AND METHODS

H and L responder Biozzi mice of selections I and
(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 ($z + w$), 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,

by latex bead injection.

Treatment with anti-asia
milliliters of effector cell su
incubated with 0.1 ml anti-a
minutes at 4°C. The cells w
RPMI-1640 without FCS, re
(Sclavo, Siena, Italy) prea
mg/ml) and spleen cells (1
dilution, incubated for 45
washed three times. The ant
a gift from K. Okumura, D
University of Tokyo.

Enrichment of Ig⁻ cells.—
obtained as described by Ma
effector spleen cells, after
shock, were incubated for
petri dishes that had been
affinity-purified goat anti-
μg/ml). Nonadherent cells,
were usually 30% of the i
routinely yielded cell popul
5% Ig^+. Adherent cells (Ig
the plates being scraped wi
cells were then washed twic

NK cell activity assay.——
was performed with the
YAC-1 and JURKAT, as ta
cells in 0.1 ml CCM w
$Na_2{}^{51}CrO_4$ (Radiochemical C
and incubated for 1 hour a
washed three times in CCM
in 96-well round-bottom
Thereafter, each well receiv
$1.25–10\times10^5$ effector splee
incubation at 37°C, the
200×g for 10 minutes, and
supernatant was measured
taneous release of ^{51}Cr radi
cultures in which effector
unlabeled target cells. Sp
tinely 5–10% of the maxi
Nonidet P40-treated labele
cytotoxicity was calculated
released in experimental an
Percent cytotoxicity=[(exp
neous release)/(maximum
lease)]×100. The percent
mean of triplicate determin

RESULTS

Longevity and Pathology

The mean life-span of

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/e mortality in H and L
ference in the initial rise
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in H and L responder
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nor is a lymphohistio-
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l with gross involvement
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volved. Morphologically,
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nder mice, develop in 30%
y in females. The sex
is statistically significant
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nat occupies the medi-
atelectatic. Invasion of
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logically, the thymus is
immature lymphocytes,

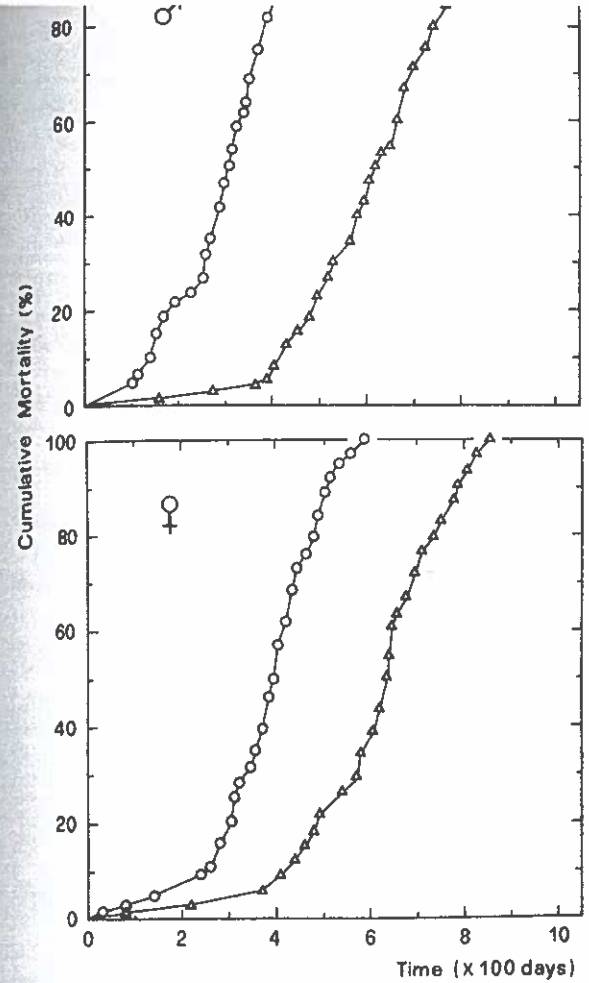
types being localized in lungs, liver, and skin. Tumors of other sites are less frequent and irregularly distributed among groups. The incidence of solid tumors is higher ($P<.001$) in females than in males of the H responder line, whereas it is similar in the two sexes of the L responder line. The incidence 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*

	H responder mice		L responder mice	
	Males	Females	Males	Females
	70	64	60	63
	613±145	611±153	305±110	386±111
	67	64	58	61
	9	8	6	19
			4	14
	9 (13)	8 (12)	10 (17)	33 (54)
'pe				
	3	6		
	2	4		
a	3	1	4	
		1		
	1	1		
			1	
	2	5		5
		1		1
	1	2		
		3		
idothelioma		3	1	
ioma		2		3

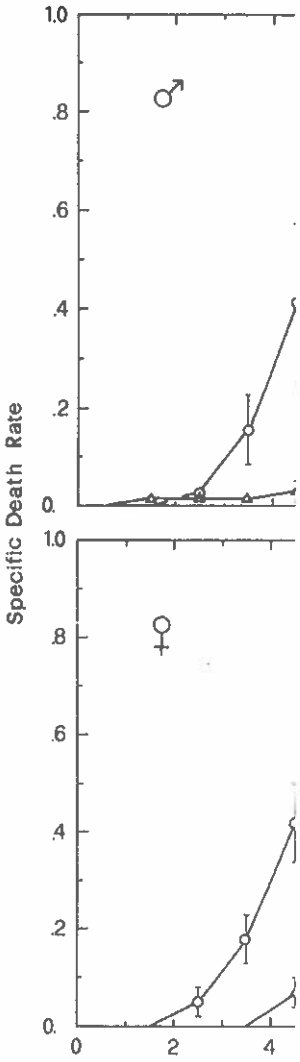


TEXT-FIGURE 1.—Cumulative mortality curves for H (Δ) and L (○) responder male and female mice of selection II.

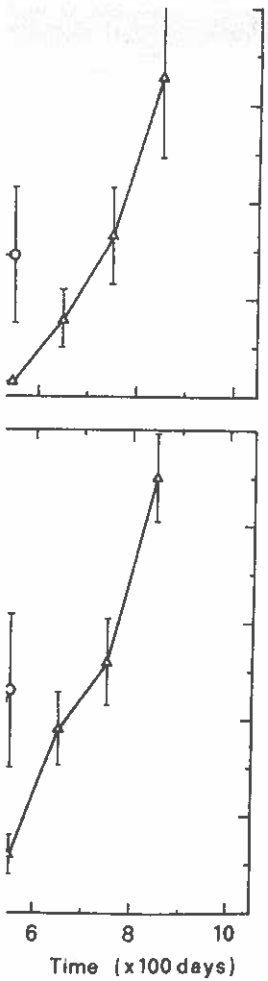
mostly in females. In contrast to the final incidence of solid tumors, which is higher in H than in L responder females (table 1), the curves of text-figure 3 indicate that the death rate from these tumors is accelerated in both sexes during the life-span of L responder mice. Thus, although H responder 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.

Lymphoma incidence is
H and L responder mic
However, the percentage
($.01<P<.05$) in males tha
sponder line. Most of the s
lungs and liver. Neoplasm
seen rarely in these mice,
cutaneous fibrosarcomas fo
females of both H and L r
is in line with previous
different mouse strains (*28*,
tumors is the same in H an
sex, and the final incidenc
than that of lymphomas i



TEXT-FIGURE 2.—Cumulative dead



· specific for solid tumors in H
emale mice of selection II as a

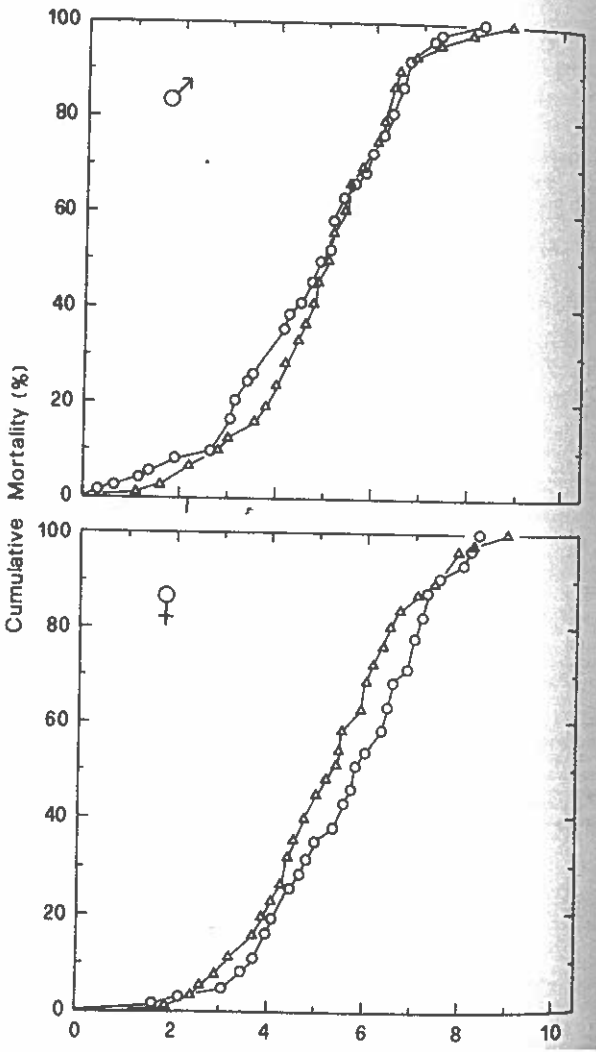
ion II. Analysis of age-
omas or solid tumors in
n III revealed complete
mortality curves for H
not shown). Altogether,
a selection III the life-
nd rate of all neoplasms
ider mice.
tive diseases also were
a selection II, their types
ily distributed between
a sexes.

om Mice of

sponder 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 antibodies 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*).



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

[a]Gastrointestinal.

finding (*24*) of shorter life
lymphomas in L respond
mice of selection I. Sinc
sponse to SRBC is a char
selections, the consistenc
thology data from both s
during selection for antib
other loci, which affect li
dence, have been random
small populations and ha
Conversely, the data fav
effect alleles for antibod
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effects on lymphoma in
associated (linkage disequ
loci with high effects on
lymphoma incidence.

The lack of any assoc
sponse to *Salmonella* fla
span and lymphoma inci
mice of selection III indic
this antibody response h
are they linked to other g
traits. Moreover, random
results of selection III.

The difference in mean
lative mortality (text-fig. 1
mice of selection II is ve
responder mice is signifi

25	50	100
11.9	15.1	21.2
17.3	20.5	21.9
10.2	14.7	21.8
11.3	15.1	19.1
12.7±1.6	*16.3±1.4*	*21.0±0.6*
2.5	4.5	6.0
2.7	3.9	6.3
2.0	3.1	7.3
3.5	4.8	8.0
2.7±0.3	*4.0±0.4*	*6.9±0.5*
17.5	26.9	30.1
19.0	26.3	27.1
23.5	37.8	36.9
24.2	36.7	49.5
21.0±1.6	*31.9±3.0*	*35.9±5.0*
16.5	23.2	18.0
8.9	11.8	17.8
17.3	30.7	34.8
7.6	9.4	9.7
12.6±2.5	*18.8±4.9*	*20.0±5.2*
5.9	10.7	16.0
7.6	10.2	14.8
6.7±0.8	*10.4±0.2*	*15.4±0.6*
12.5	16.5	25.6
13.7	14.1	19.0
13.1±0.6	*15.3±1.2*	*22.3±3.3*
17.0	8.2	28.2
15.0	20.0	39.0
16.0±1.0	*14.1±5.9*	*33.6±5.4*
18.0	26.4	43.0
29.2	37.3	61.6
23.6±5.6	*31.9±5.4*	*52.3±9.3*
3.7	4.1	7.2
3.2	3.6	5.3
4.0	4.6	7.9
3.6±0.2	*4.1±0.3*	*6.8±0.8*
15.0	17.4	22.0
13.0	16.2	20.3
13.0	15.2	21.1
13.7±0.7	*16.3±0.6*	*21.1±0.5*

ous release of ^{51}Cr (cpm) were
1,016 and 78; expt 2, 513 and
d 66; expt 2, 447 and 150. In

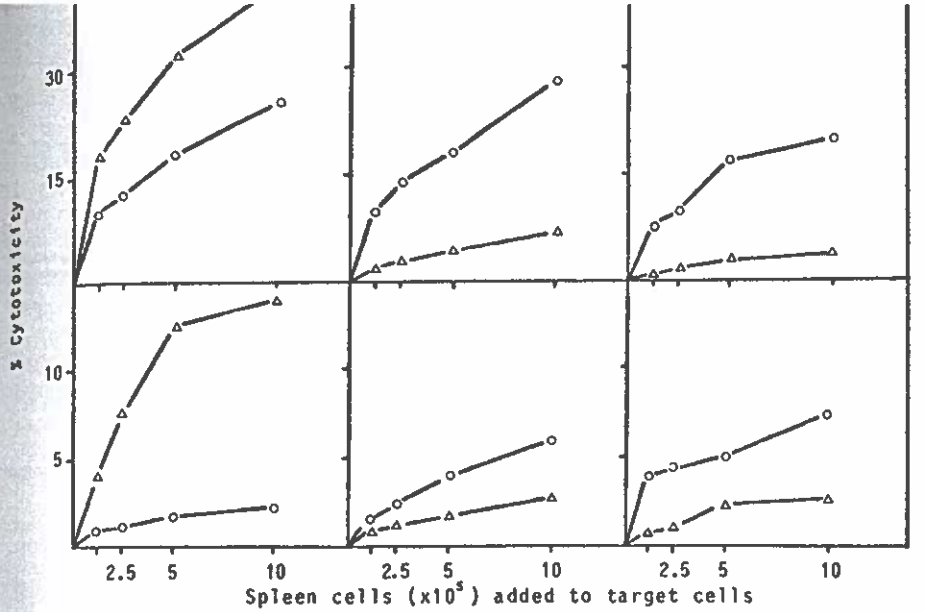
SE.

life-span between L and
n II appears to reflect the
of lymphomas (text-fig. 2)
3). Indeed, both cancers
aster development in L
sponder mice of selection

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



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 ap-

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

Selection	Life-span	Lymphoma incidence	NK activity

peared much later (text-f
latent period NK cells
and/or activity which m
younger animals and m
immunity. Evaluation of
lated aged H and L respo
laboratory. Also, correlati
incidence of benzo[*a*]pyre
H and L responder mic
information to this contro

In conclusion, results f
firm and extend our pr
from selection I (*24*), tog
tion III, indicate that g
responsiveness to some b
may affect longevity and
selection II L responder
spans and higher lympho
H responder mice, but
between L and H respond
NK cell activity was assess
mice, it was found to
responder mice of selectio
responder mice of both
findings indicate that lo
dence at death are indepe
mice selected for H or L
natural antigens.

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