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A Simple Index for the Measurement of the Runting Syndrome and Its Use in the Study of the Influence of the Gut Flora in its Production

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Summary. This communication describes a simple, sensitive index for the measurement of the runting syndrome. Evidence is presented that the bacterial flora of the gut and endotoxin derived therefrom may play a role in induction of the runting syndrome. There was no evidence of bacterial invasion of runted animals. Neomycin sulphate given by gavage reduced the intensity of the runting syndrome. Furthermore, bacterial endotoxin produced runts sharing many of the features found in runts produced by the graft-*versus*-host reaction, cortisone acetate, bacterial vaccine, neonatal reovirus 3 infection and neonatal thymectomy.

INTRODUCTION

Many experimental treatments produce clinically obvious runting (Keast, 1968). Classically this syndrome has been associated with the graft-*versus*-host reaction (Billingham and Brent, 1957; Simonsen, 1957; Russell, 1960; Nisbet and Heslop, 1962; McBride, 1966). Comparison of runted animals produced by other means with the conventional graft-*versus*-host reaction runt has suggested that there is an underlying immunological aetiology in all runts.

Further analysis however suggests that lesions, other than those present in the lymphoid system and the reticulo-endothelial system, may not be the result of immunological reactions (Hilgard, Martinez and Good, 1965; McBride, 1966; Keast, 1968). Furthermore, lesions in the lymphoid system do not necessarily indicate that direct immunological reactions are involved in the production of lymphoid hypoplasia. It would be useful to employ a sensitive method of measuring animals entering into the clinical phase of the syndrome. The runts may then be defined and certain other criteria used to decide whether, in the case of a suspected immunological aetiology, lymphoid hyper- or hypoplasia, involution of lymph nodes, or other acceptable phenomena are present. As further study of the runted animal may be desirable, the method must not affect the animal in any way.

This communication describes a simple, sensitive index for the measurement of the runting syndrome, and presents evidence that the bacterial flora of the gut is implicated. An accompanying communication on the histopathology supports this view.

MATERIALS AND METHODS

The runting index (RI)

Groups of animals under test and groups of normal animals of the same age and sex may be weighed and the group means ($\bar{x}$) calculated.

Thus let: Mean weight of test animals = $T\bar{x}$;
Mean weight of normal animals = $N\bar{x}$;
Runting index = RI;
then: $T\bar{x}-N\bar{x}$ = RI.

The normal mouse index is the difference between the weights of two normal groups of mice at any given age. The limits of the normal mouse index are the sum of the two 95 per cent confidence limits of the standard error of the mean ($2\times\pm1{\cdot}96$ SE$\bar{x}$). Similar limits apply to the RI, but for all practical purposes groups of animals producing RI values falling below $2\times-1{\cdot}96$ SE$\bar{x}$ can be classified as exhibiting a runting syndrome. Groups of animals producing RI values above $2\times+1{\cdot}96$ SE$\bar{x}$ may be considered to have had their growth significantly enhanced following the experimental treatment. The limits of the RI are not presented in the figures, however a maximum of ±1 RI unit over the range tested has been encountered and may be applied to all test groups.

The following situations known to produce the runting syndrome were tested:

The graft-versus-*host reaction* (Russell, 1960)

Spleen cells from adult mice of the Royal Perth Hospital strain of albino mice were prepared in Hanks's buffered salt solution. One million to 10^7 viable cells were inoculated intraperitoneally (i.p.) into neonatal Prince Henry (PH) mice.

Cortisone injection into neonates (Duhig, 1965)

A suspension of cortisone acetate was made in physiological saline and 0·1 mg of cortisone acetate/g of mouse was inoculated i.p. into neonatal PH mice.

Neonatal thymectomy (Miller, 1960)

PH mice less than 24 hours of age were thymectomized while under hypothermic anaesthesia. Partially thymectomized animals were controls.

Bacterial vaccine injection into neonates (Ekstedt and Nishimura, 1964)

Staphylococcus aureus strain E49 was grown overnight at 37° on MIA plates and collected in 0·3 per cent formalin. All preparations of vaccine were tested for sterility before use. Approximately 10^9 organisms were inoculated i.p. into neonatal PH mice at 48-hourly intervals through the course of the experiment up to 30 days. Control animals received 0·3 per cent formalin solution. A heat killed saline suspension of the same organism was prepared and used, and essentially the same results were produced. Data on these animals are not presented.

Bacterial endotoxin injection into neonates

One hundred and thirty micrograms of *E. coli* 0111: B4 endotoxin (Difco) in pyrogen free water or a crude preparation prepared from organisms grown from the PH mouse gut was inoculated i.p. every 48 hours into PH mice up to 11 days of age. This was increased to 260 μg at 48-hourly intervals from 11 to 30 days.

Reovirus 3 *infection of neonatal mice*

As the results obtained by Walters, Joske, Leak and Stanley (1963) were obtained from PH mice, the RI values calculated from these results are also directly applicable in this section.

Neomycin sulphate treatment

Preliminary trials on the extent to which neomycin sulphate could be tolerated by young mice and effectively used for the control of the gut flora of the PH mice gave the following pertinent information:

Control of bacterial flora

In order to obtain efficient control over the bacterial flora of the gut, both the mother and the litter had to be gavaged at regular intervals. The litter became colonized by Gram-negative flora within 24 hours of birth. However, if the mother was immediately gavaged *post partum* and the cage changed after 24 hours, then daily gavaging of the mother (0·2 ml, 0·66 mg/ml neomycin sulphate) and gavaging of the litter (commencing at day 3) on alternate days, was most effective in controlling gut population and reduced coliform counts to a minimum. Initially a drop of 0·66 mg/ml neomycin sulphate was given orally to the litters followed by gavaging with volumes up to 0·1 ml (0·66 mg/ml) neomycin sulphate. Young mice for the most part were kept free of detectable coliforms up to 12–16 days of age, but when the young began to leave the nest they all became colonized to some extent by coliforms.

In the case of thymectomized animals, neomycin sulphate was added to the drinking water at a concentration of 4·0 mg/ml. This reduced the gut coliform count substantially, but coliform colonization was not completely prevented. Furthermore, *Streptococcus faecalis* was present in all faecal samples tested, but was not a neomycin sulphate resistant strain.

Effect on the blood leucocytes

It was found that neomycin sulphate was freely absorbed through the gut in neonatal PH strain mice, however this gut permeability no longer existed at 3 days of age. Blood leucocyte changes did occur.

Further tests on 210 animals ranging from 6 to 30 days in age suggested that leucocyte counts, although variable, were not the direct result of the neomycin sulphate gavaging. Rarely an apparent gut permeability to neomycin sulphate was encountered at 6 days of age, and once at 15 days of age. This apparent permeability of the gut to neomycin sulphate may be explained by a chance overflow of the inoculum into the lungs and hence the blood stream. This was not considered to be of experimental significance; however, no experimental animals received neomycin sulphate before 3 days of age, or more often than once in a 48-hour period.

Animals were weighed on a Mettler balance and arithmetic means calculated. Initially groups of animals exceeded 30 per treatment, except for the thymectomy section, where only nine animals made up the group.

Experimental design

The experiments were divided as follows:

Neomycin sulphate treatments and treatments to produce endotoxin and bacterial vaccine runts were carried out as required for 30 days. From 30 to 60 days no treatments

were given and this may be considered as a potential recovery period. This period was incorporated into the experimental design to ascertain whether the runting induced by the treatments in fact gave permanently runted animals, or whether these runts were still able to return to within the normal weight range once the experimental treatment had been removed.

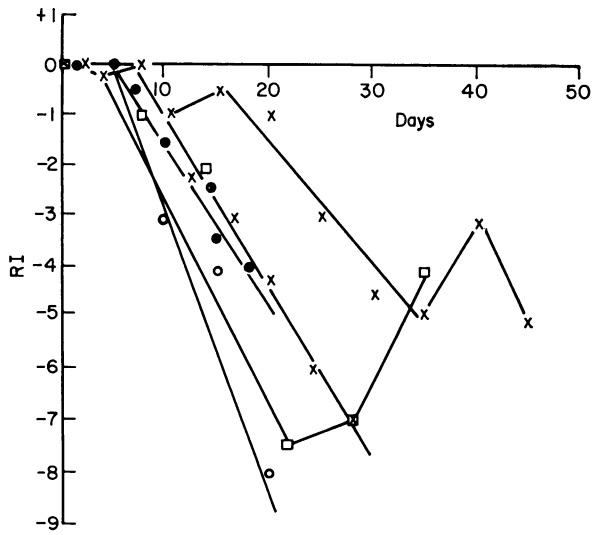
The thymectomy section was designed as follows:

At day 25 the thymectomized mice were divided into three groups:
Group 1, untreated controls: nine animals.
Group 2, neomycin sulphate in drinking water (4 mg/ml): six animals.
Group 3, neomycin sulphate in drinking water plus endotoxin i.p. on alternate days: seven animals.
A further group of partially thymectomized controls (five animals) was also included.

RESULTS

ANALYSIS OF RUNTING ANIMALS ALREADY RECORDED IN THE LITERATURE

In order to test whether the RI has a general application, the literature was surveyed and a series of results already accepted as having illustrated the runting syndrome were



Symbol	Runts	Author	Regression	Slope	Significance
●	GvHR	Russell	$y = 1{\cdot}68 - 0{\cdot}32x$	−0·32	0·001
+	Reovirus	Stanley	$y = 2{\cdot}37 - 0{\cdot}34x$	−0·34	0·001
○	Cortisone	Duhig	$y = 3{\cdot}17 - 0{\cdot}56x$	−0·56	0·01
□	Bacterial vacc.	Ekstedt	$y = 0{\cdot}80 - 0{\cdot}35x$	−0·35	0·05
×	Thymectomy	Duhig	$y = 3{\cdot}26 - 0{\cdot}24x$	−0·24	0·01

FIG. 1. The RI calculated and graphed from data already presented in the literature as illustrative of the runting syndrome. Regressions have been calculated and with the levels of significance are presented in the key.

converted to RI values and the results are plotted in Fig. 1. Linear regressions apparent during the maximum phase have been calculated and shown to be statistically significant.

The calculations were based on data obtained in mice for runting induced by:

(1) Graft-*versus*-host reaction (G*v*HR)—Russell (1960).
(2) Cortisone acetate—Duhig (1965).
(3) Neonatal thymectomy—Duhig (1965).
(4) Bacterial vaccine—Ekstedt and Nishimura (1964).
(5) Neonatal infection with reovirus—Walters *et al.* (1963).

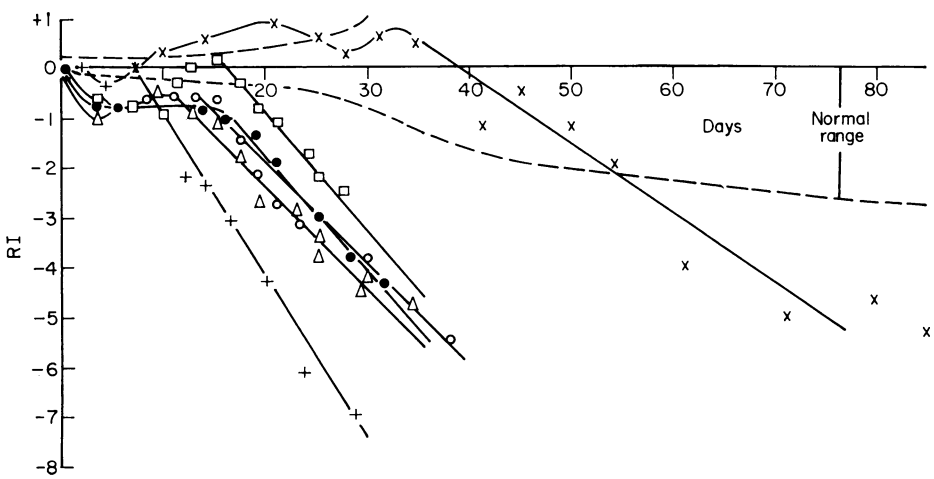
The results show that the RI is applicable to these situations (Fig. 1).

ANALYSIS OF PH STRAIN MICE RUNTED BY VARIOUS METHODS

Results were converted into RI values and plotted in Fig. 2. Linear regressions apparent during the maximum phase have been calculated and all are statistically significant.

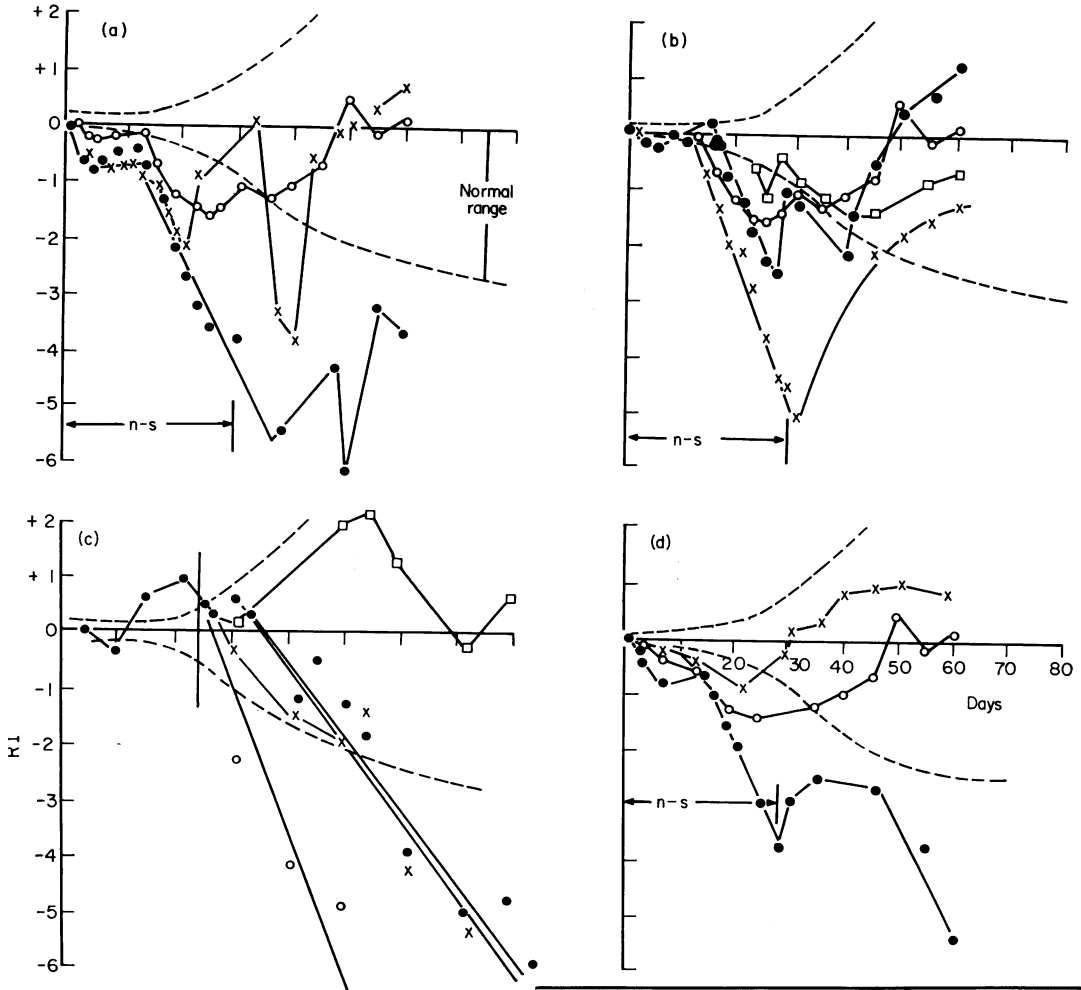
ANALYSIS OF THE EFFECTS OF NEOMYCIN SULPHATE TREATMENT ON THE COURSE OF THE RUNTING SYNDROME

At the same time and in conjunction with results obtained in the previous section, further groups of animals were given the various 'runt-inducing' treatments and were used to



Symbol	Runts	Regression	Slope	Significance
●	G*v*HR	$y = 2{\cdot}72 - 0{\cdot}23x$	−0·23	0·001
+	Reovirus	$y = 2{\cdot}37 - 0{\cdot}34x$	−0·34	0·001
○	Cortisone	$y = 1{\cdot}61 - 0{\cdot}19x$	−0·19	0·001
□	Bacterial vacc.	$y = 3{\cdot}41 - 0{\cdot}22x$	−0·22	0·001
×	Thymectomy	$y = 5{\cdot}01 - 0{\cdot}13x$	−0·13	0·001
△	Endotoxin	$y = 1{\cdot}25 - 0{\cdot}18x$	−0·18	0·001

FIG. 2. The RI calculated and graphed from data obtained on the runting syndrome produced in the PH strain of mice by several methods. Regressions have been calculated and with the regression coefficients (r) are presented in the key.



Graph	Symbol	Treatments
(a)	●	Cortisone
	×	Cortisone + neo. sulphate
	○	Neo. sulphate
(b)	●	Bact. vaccine
	×	Bact. vaccine + neo. sulphate
	○	Neo. sulphate
	□	Formalin 0·3 per cent
(c)	●	Neo. thymectomy
	×	Neo. thymectomy + neo. sulphate
	○	Neo. thymectomy + endotoxin + neo. sulphate
	□	Partial thymectomy
(d)	●	GvHR
	×	GvHR + neo. sulphate
	○	Neo. sulphate
(e)	○	260 μg endotoxin/48 hours
	×	26·0 μg endotoxin/48 hours
	●	2·60 μg endotoxin/48 hours
	□	0·26 μg endotoxin/48 hours

FIG. 3. The RI is presented for the runting syndrome produced in the PH strain of mice by several methods. The modifications to this following neomycin sulphate gavaging of test animals are also presented. (a) Cortisone runts, (b) bacterial vaccine runts, (c) thymectomy runts, (d) GvHR runts, and (e) endotoxin runts.

obtain information on the effect of neomycin sulphate gavaging on the course of the development of the RI.

Results were converted into the RI and are presented graphically in Fig. 3.

Neomycin sulphate has been shown to modify the RI of certain runted animals. In the graft-*versus*-host reaction the RI was much reduced and by 25 days of age animals were within the limits of the normal mouse index. They remained within these limits up to 60 days. Furthermore, less than 20 per cent of the animals survived 70 days in the graft-*versus*-host reaction groups, whereas 75 per cent of the animals survived to 70 days in the graft-*versus*-host reaction group receiving neomycin sulphate gavaging (Fig. 3).

In the case of cortisone runts, neomycin sulphate again enhanced survival. The neomycin sulphate treatment was stopped at 30 days and at 36 days the RI became statistically significant, indicating a newly acquired sensitivity of the animals, presumably to some factor that was controlled by neomycin sulphate. This sensitivity was transient and animals subsequently returned to within the normal limits of the RI and 89 per cent survived to 60 days, whereas less than 30 per cent of untreated animals survived to this time (Fig. 3).

Neomycin sulphate gavaging had the reverse effect on the RI of bacterial vaccine runts, and gavaging increased the RI and the percentage dying (Fig. 3). Although there was no measurable effect on the RI for endotoxin runts or reovirus 3 runts, more animals died in group receiving neomycin sulphate treatment. A few reovirus runts surviving longer than 30 days still remained sensitive to the neomycin sulphate treatment.

Neomycin sulphate had little effect on the RI of thymectomized animals; however, neomycin sulphate plus bacterial endotoxin enhanced the development of the RI and animals also died earlier (Fig. 3).

The titration of endotoxin from *E. coli* 0111:B4 indicated that levels as low as 2·6 μg/48 hours given IP can produce significant runting as measured by the RI in the PH strain of mice (Fig. 3).

DISCUSSION

A simple and effective method—the runting index (RI) has been defined and applied to results already published and accepted as illustrating the runting syndrome (Fig. 1). It should be noted that the RI measures both animals that fail to grow normally and animals which actually lose weight. The RI has also been shown to be applicable to all runted animals developed in one strain of mice where fiducial limits are defined (Figs. 2 and 3). A runting index has been proposed recently by Festenstein, Abrahams and Bokkenheuser (1967) for rabbits. However, statistical limits for the normal animals range do not exist and the test becomes meaningless. The results reported here illustrate the multiplicity of agents which cause runting.

The results from the cortisone-treated runts are in agreement with those of Duhig (1965), where terramycin was shown to have a beneficial effect on survival of runts produced by the cortisone acetate treatment. Furthermore, the work of Reed and Jutila (1965) has shown that germ-free animals, while still runting, have a higher survival rate than conventional animals following cortisone treatment. They suggest an important role for the gut flora in runting syndromes. Similarly, more graft-*versus*-host reaction runts survive and return to within the normal RI range after neomycin sulphate treatment, as shown by van Bekkum, van Putten and de Vries (1962).

Both these sets of results suggest that at least the lethal threshold for the treatments is raised if the gut flora are controlled by neomycin sulphate. Histopathological changes in animals receiving the neomycin sulphate are also less acute (Keast and Walters, 1968).

The increased RI of bacterial vaccine runts (Fig. 3) given neomycin sulphate is difficult to explain. There may be an increase in concentration of gut endotoxin due to the killing of the natural flora, but this would be present in the other treatments. The gut in bacterial vaccine runts may show histopathological changes which, under our experimental conditions, are more pronounced in this group of animals than in either the graft-*versus*-host reaction runts or cortisone runts. Thus an increased permeability to gut flora endotoxin, plus a possible neomycin sulphate toxicity, could explain the results. The apparent sensitivity to neomycin sulphate is also present in endotoxin runts and reovirus 3 runts, but is represented mainly as an increase in the number of deaths. No further histopathological changes are found in bacterial vaccine or endotoxin runts after neomycin sulphate (Keast and Walters, 1968). Multiple neomycin sulphate gavaging alone, while slightly retarding early growth, does not induce lesions under our experimental conditions.

Bacterial isolations from the blood of several of the clinically ill animals were attempted and proved to be negative, while the pathological changes in animals which had received neomycin sulphate were less striking than those for untreated runts, except for endotoxin and bacterial vaccine runts (Keast and Walters, 1968).

The results suggest that bacterial endotoxin can play an important role in this syndrome, irrespective of the method of induction. This has been implied in the literature on conventional and germ-free animals by Howard, Biozzi, Halpern, Stiffel and Moreton (1959); Böhme and Bouvier (1960); Howard (1961); Parrott (1962); Brooke (1964); McIntire, Sell and Miller (1964); Miller and Howard (1964); Wilson, Sjodin and Bealmear (1964); Duhig (1965); Read and Jutila (1965); Safford and Jutila (1965); McBride (1966); Ekstedt and Hayes (1967); Keast (1968). Whether the animals become more sensitive to normal levels of endotoxin, as suggested by the early work of Howard *et al.* (1959); Böhme and Bouvier (1960) and Howard (1961), or whether more endotoxin passes through an experimentally damaged gut to cause these effects is unknown.

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REFERENCES

Billingham, R. E. and Brent, L. (1957). 'A simple method for inducing tolerance of skin homografts in mice.' *Transplant. Bull.*, **4**, 67.

Böhme, D. and Bouvier, C. A. (1960). 'Influence of killed *Mycobacterium tuberculosis* (BCG) and its constitutents upon the reticuloendothelial system, internal organs and percentage distribution of leukocytes in neonatal albino mice.' *Reticuloendothelial Structure and Function* (Ed. by J. H. Heller), p. 165. Ronald Press, New York.

Brooke, M. S. (1964). 'Experimental runt disease in mice caused by *Salmonella typhimurium* var. copenhagen.' *J. exp. Med.*, **120**, 375.

Duhig, J. T. (1965). 'The beneficial effects of oxytetracycline in cortisone-induced wasting disease.' *Nature* (*Lond.*), **207**, 651.

Ekstedt, R. D. and Hayes, L. L. (1967). 'Runt diseases induced by non-living bacterial antigens.' *J. Immnuol.*, **98**, 110.

Ekstedt, R. D. and Nishimura, E. T. (1964). 'Runt

disease induced in neonatal mice by sterile bacterial vaccines.' *J. exp. Med.*, **120**, 795.

FESTENSTEIN, H., ABRAHAMS, C. and BOKKENHEUSER, V. (1967). 'Runting syndrome in neonatal rabbits infected with *Treponema pallidum*.' *Clin. exp. Immunol.*, **2**, 311.

HILGARD, H. R., MARTINEZ, C. and GOOD, R. A. (1965). 'Production of runt disease in tolerant mice by injection of syngeneic lymphoid cells.' *J. exp. Med.*, **122**, 1017.

HOWARD, J. G. (1961). 'Increased sensitivity to bacterial endotoxin of F_1 hybrid mice undergoing graft-*versus*-host reaction.' *Nature* (*Lond.*), **190**, 1122.

HOWARD, J. G., BIOZZI, G., HALPERN, B. N., STIFFEL, C. and MORTEN, D. (1959). 'The effect of *Mycobacterium tuberculosis* (BCG) infection on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection.' *Brit. J. Path.*, **40**, 281.

KEAST, D. (1968). 'Runting syndromes, autoimmunity and neoplasia.' *Adv. Cancer Res.*, **11** (in press).

KEAST, D. and WALTERS, M. N.-I. (1968). 'The pathology of murine runting and its modification by neomycin sulphate gavages.' *Immunology*, **15**, 247.

MCBRIDE, R. A. (1966). 'Graft-*versus*-host reaction in lymphoid proliferation.' *Cancer Res.*, **26**, 1135.

MCINTIRE, K. R., SELL, S. and MILLER, J. F. A. P. (1964). 'Pathogenesis of the post-neonatal thymectomy wasting syndrome.' *Nature* (*Lond.*), **204**, 151.

MILLER, J. F. A. P. (1960). 'Studies on mouse leukemia. Leukemogenesis by cellfree filtrates inoculated in newborn and adult mice.' *Brit. J. Cancer*, **14**, 83.

MILLER, J. F. A. P. and HOWARD, J. G. (1964). 'Some similarities between the neonatal thymectomy syndrome and GvH disease.' *J. Reticulo-endothelial Soc.*, **1**, 369.

PARROTT, D. M. V. (1962). 'Strain variation in mortality and runt disease in mice thymectomised at birth.' *Transplant. Bull.*, **29**, 474.

NISBET, N. W. and HESLOP, B. F. (1962). 'Runt disease.' I. and II. *Brit. med. J.*, **i**, 129 and 206.

REED, N. D. and JUTILA, J. W. (1965). 'Wasting disease induced with cortisol acetate: Studies in germ-free mice.' *Science*, **150**, 356.

RUSSELL, P. S. (1960). 'The weight-gain assay for runt disease in mice.' *Ann. N.Y. Acad. Sci.*, **87**, 445.

SAFFORD, J. W., Jr and JUTILA, J. W. (1965). 'Studies on the etiology of the runting syndrome in mice.' *Fed. Proc.*, **24**, 637.

SIMONSEN, M. (1957). 'The impact on the developing embryo and newborn animal of adult homologous cells.' *Acta path. microbiol. scand.*, **40**, 480.

VAN BEKKUM, D. W., VAN PUTTEN, L. M. and DE VRIES, M. J. (1962). 'Anti-host reactivity and tolerance of the graft in relation to secondary disease in radiation chimeras.' *Ann. N.Y. Acad. Sci.*, **99**, 505.

WALTERS, M. N-I., JOSKE, R. A., LEAK, P. J. and STANLEY, N. F. (1963). 'Murine infection with reovirus. I. Pathology of the acute phase.' *Brit. J. exp. Path.*, **44**, 427.

WILSON, R., SJODIN, K. and BEALMEAR, M. (1964). 'The absence of wasting in thymectomixed germfree (axenic) mice.' *Proc. Soc. exp. Biol.* (*N.Y.*), **117**, 237.