J. Hyg., Camb. (1974), **72**, 379
Printed in Great Britain

Determination of the colonization resistance of the digestive tract of individual mice

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(*Received* 20 *September* 1973)

SUMMARY

It has been shown that it is possible to investigate the colonization resistance in individual mice by determining the concentration of a certain contaminant (S.R.-*E. coli*) in the faeces during the first 4 days after contamination. Experimental contamination is contra-indicated in many cases such as in individuals with decreased resistance to infection. Particularly in this group, the value of the colonization resistance should be determined. It appeared to be possible to determine the colonization resistance in such individuals by quantitative biotyping of the Enterobacteriaceae species in the faeces on several consecutive days.

INTRODUCTION

The determination of colonization resistance (CR) of the digestive tract to pathogenic and potentially pathogenic (p.p.) bacteria in groups of animals is now a well-established technique that has contributed to the rational design of isolation methods in animal research.

We showed previously that the CR in mice is controlled by anaerobic bacteria which perform their activity directly by 'ecological interaction' and indirectly via the host (van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971). An important aspect of the indirect activity is the stimulation of intestinal peristaltic activity by the microflora (Abrams & Bishop, 1966).

So far, colonization resistance has been measured by experimental contamination of mice and has been defined as the log of the oral dose of a specific bacterium that results in colonization of the digestive tract for 2 weeks or longer in 50% of a group of 20 animals (van der Waaij *et al.* 1971).

The measurement of CR in a group of mice is helpful in determining the extent to which isolation precautions must be provided in order to maintain the group free of potentially pathogenic bacteria. This technique does not provide specific information about the CR of individuals within a group. Such information is essential if the individual has a decreased resistance to infection and must be isolated in order to prevent development of infections from exogenous micro-organisms.

A possible method for determining individual CR was suggested by the finding that, after oral administration to mice of a single dose of an Enterobacteriaceae

species, the population density of these bacteria in the intestinal tract varied inversely with the CR. This study was undertaken to determine the nature of this inverse relationship. In addition, the concentration of other Enterobacteriaceae species in the faeces and the composition of the gram negative microflora was determined. This was done in order to investigate the possibility of the use of endogenous bacteria for the determination of CR. Total body irradiation, and antibiotic decontamination of the digestive tract were used alone or in combination in order to decrease the CR, while various contaminating doses of *Escherichia coli* were investigated.

MATERIALS AND METHODS

Mice

Conventional ND_2 female mice 8–12 weeks of age, weighing 28–35 g., were used.

Housing

The mice were housed one per cage in autoclaved macralon cages. The cages with antibiotic-treated animals were maintained in a laminar crossflow bench to prevent airborne contamination (van der Waaij & Andreas, 1971). Sterilized food and drinking water were supplied *ad libitum* to all animals.

Antibiotics

The mice that were decontaminated were treated with antibiotics. These were supplied *ad libitum* in drinking water containing 5 g. of neomycin, 5 g. of streptomycin, 5 g. of bacitracin and 0·1 g. of pimaricin/l. After the first week of treatment when stool and oral cultures were sterile, the concentration of streptomycin and bacitracin was reduced by 50 %, and use of neomycin was discontinued. Treatment with this low-dose regimen was continued throughout the experiment.

Oral contamination

The contaminating dose of a streptomycin-resistant (S.R.) strain of *Escherichia coli* was prepared by diluting an overnight broth culture with fresh broth to the desired concentration.

Conventional animals

Four groups of 8 mice were contaminated orally with doses of 10^3, 10^5, 10^7, 10^9 or 10^{11} S.R.-*E. coli* suspended in 0·1 ml. of broth. This procedure was repeated four times; 32 animals were thus exposed to each oral dose.

Antibiotic-treated animals

A contamination schedule identical with that for conventional animals was used. Two additional groups of 8 mice that received 10^2 bacteria were added. The bacteria were administered 10 days after the start of antibiotic treatment. The reasons for waiting 10 days were to assure 'physiologic stability' and complete elimination of neomycin from the g.i. tract (van der Waaij, 1969).

Irradiated animals

A contamination schedule identical with that for conventional mice was used. However, instead of 4 groups of 8 mice, only 2 groups were used for each dose. Contamination was performed at day 4 after irradiation.

Sampling

Inasmuch as the spread in the log of the concentration of S.R.-*E. coli* in faecal samples initially collected 3 times daily was very small (in no case more than 1 log), only one daily collection was used. After oral contamination, fresh faeces were collected on days 1, 2, 3, and 4. In the experiments with the unirradiated conventional mice, collections were also made on days 7 and 14. A 50 mg. sample of faeces was suspended in 0·5 ml. brain heart infusion broth (DIFCO). Subsequently, serial dilution was performed in trays in steps of 1:11. The trays contained 0·5 ml. of plain brain heart broth per cup or 0·5 ml. of broth to which streptomycin (100 μg./ml.) had been added. The streptomycin broth was employed when it was desired to isolate only streptomycin-resistant *E. coli*. Dilution was accomplished by the use of diluting loops (Flow labs) of 0·05 ml. capacity. Eight dilution steps routinely were carried out for each sample. When higher concentrations were expected, serial dilution was performed in 12 dilution steps. After overnight incubation at 37° C., the cultures were subcultured on Endo agar (DIFCO) for isolation, pure culturing, and biotyping.

Biotyping

With the help of 19 different fermentation reactions, the Enterobacteriaceae species isolated from the faeces of 4 conventional irradiated and 4 conventional unirradiated mice were typed. Details of the typing technique were described in an earlier publication (van der Waaij, Speltie & Vossen, 1972). Biotyping of the aerobic gram negative faecal bacteria was performed twice weekly for 4 subsequent weeks in the unirradiated mice and until death in the irradiated animals. Inventory of the faeces by biotyping the Enterobacteriaceae species isolated was started at the day on which the animals were experimentally contaminated. The mice belonged to the groups which received an oral dose of 10^5 S.R.-*E. coli* cells.

Biotypes which were isolated only once are designated as 'contaminations'; those which were isolated from two or more subsequent samples are indicated as 'colonizations'.

Irradiation

The animals received total body irradiation in perspex cages under conditions of maximal back scatter. Irradiation was performed with a Philips Muller X-ray machine at 300 kV, 10 mA with an HVL of 3 mm. Cu. The applied irradiation dose was 700 rad. For the conventional animals used in this study, this dose represents an LD 100. It results in severe bone marrow damage and in the first day(s) after irradiation some mucosal damage to the intestines (Gordon, Ruml, Hahne & Miller, 1955). Groups of 16 mice were contaminated with the same doses of S.R.-*E. coli* as were given to the unirradiated conventional animals. Contamination was

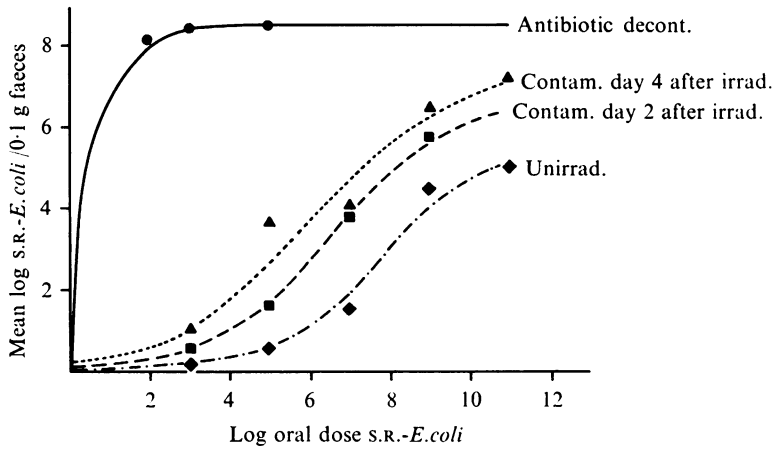

Fig. 1. Relation between the oral dose of S.R.-*E. coli* and its concentration in the faeces after contamination of antibiotically decontaminated, irradiated, and unirradiated conventional mice.

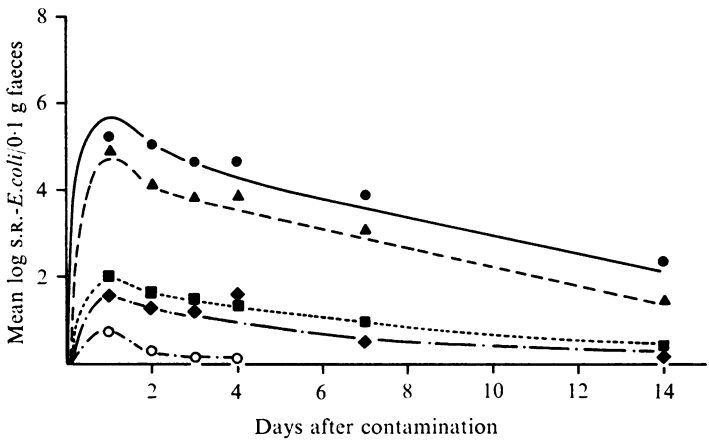

Fig. 2. Mean* faecal concentration of S.R.-*E. coli* at various intervals after contamination in conventional unirradiated mice. Oral dose of S.R.-*E. coli*: ●, 10^{11}; ▲, 10^{9}; ■, 10^{7}; ◆, 10^{5}; ○, 10^{3}. * 32 mice/dose.

performed on the second day after irradiation. In similar experiments, contamination was performed on day 4 after irradiation. The antibiotic-treated animals were irradiated under isolated conditions.

RESULTS

The mean faecal concentration of S.R.-*E. coli* during the first 4 days after contamination in conventional irradiated and unirradiated mice varied directly with the dose (Fig. 1). In the unirradiated animals, the peak concentration occurred within 1 day and was followed by a gradual reduction (Fig. 2). In the irradiated mice, a peak concentration at day 1 was only seen after the lowest dose of 10^{3} bacteria (Fig. 3). After the contaminations with higher doses, no peaks were seen,

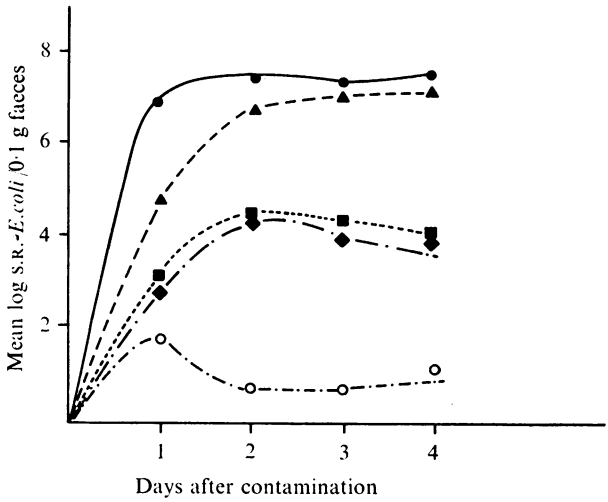

Fig. 3. Mean* faecal concentration of S.R.-*E. coli* at various intervals after contamination in conventional irradiated mice. Oral dose of S.R.-*E. coli*: ●, 10^{11}; ▲, 10^{9}; ■, 10^{7}; ◆, 10^{5}; ○, 10^{3}. * 16 mice/dose.

while considerably higher concentrations of bacteria /g. of faeces were seen than in the unirradiated mice.

The relation between the log of the oral dose of contaminating bacteria and the log of the mean concentration of S.R.-*E. coli* in the faeces is illustrated in Fig. 1. The mean concentrations are calculated from the data obtained in the first 4 days after contamination. This is the most likely period for colonization to occur even after low contaminating doses of bacteria (van der Waaij, de Vries & Lekkerkerk, 1972). Antibiotic-decontaminated mice were shown to have a concentration of approximately 10^{8} bacteria/0·1 g. of faeces following doses as low as 100 cells, whereas unirradiated conventional mice never exceeded a mean concentration of 10^{6} bacteria/0·1 g., even when 10^{11} bacteria were administered. Furthermore, at lower doses of 10^{3} S.R.-*E. coli* cells and, presumably even up to 10^{4} cells, no measurable concentrations were found in the faeces of most mice. The shape of the dose–faecal concentration curve for irradiated mice was similar to that for unirradiated mice, but the mean S.R.-*E. coli* concentrations in the faeces were higher. These results indicate that elimination of the microflora by antibiotic decontamination has a much more drastic effect on the CR than does lethal irradiation. For every interval after contamination (days 1, 2, 3, 4, 7 and 14), the percentage of positive samples and the concentrations of the contaminant in the samples were determined. These determinations were made without reference to the contaminating dose. For each of the 6 periods, a linear correlation was demonstrated between the mean log concentration of S.R.-*E. coli* in the faeces of individual animals within a group and the percentage of the group excreting S.R.-*E. coli* (Fig. 4). Inasmuch as the standard deviation of the mean log concentration of S.R.-*E. coli* in the faeces was shown to be very small (S.D. varied between 0·20 and 0·32) (Fig. 4), it is possible to relate individual concentrations to the percentage of animals excreting the contaminant. Fig. 4 also indicates that the level of CR, expressed as the percentage

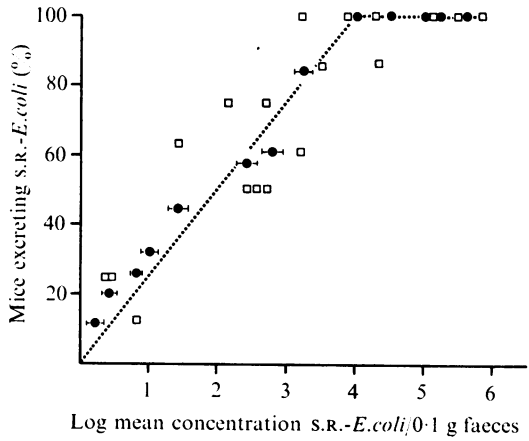

Fig. 4. Relation between the mean* concentration of S.R.-*E. coli* in the faeces 1–14 days after oral contamination and the percentage of mice that excreted the contaminant. * Unirradiated 32 mice/dose; irradiated 16 mice/dose. □, Mean irradiated mice (8 animals/group); ⊢●⊣, mean and S.D. unirradiated mice (80 animals/group).

Table 1. *Quantitative and qualitative results of biotyping of Enterobacteriaceae species following contamination with* S.R.-E. coli *in conventional irradiated and unirradiated mice**

Condition mice	Log oral dose S.R.-*E. coli*	Mean log conc./0·1 g. faeces in first 4 days: Contaminant (S.R.-*E. coli*)	Mean log conc./0·1 g. faeces in first 4 days: Other gram negative bacteria	Biotyping results gram negative bacteria: Mean no. cont./sample	Mean no. colon./sample	Colon./contam.
Conventional unirradiated	5	4·2	4·1	0·57	0·20	0·35
		4·1	3·5	0·14	0	0
		4·7	4·6	0·29	0	0
		4·5	4·3	0·49	0·20	0·40
Conventional contaminated at day 2 after irradiation	5	4·2	6·5	1·14	0·80	0·70
		5·2	7·1	0·85	0·80	0·94
		5·0	6·2	1·43	1·00	0·70
		4·7	6·1	0·57	0·60	1·05

* 4 animals/group.

of mice that excrete the contaminant is independent of the dose in which the contaminant was administered and of the time interval after contamination.

The biotyping results (Table 1) show a relation between the concentration of the various biotypes in the faeces and the mean concentration of the contaminant (S.R.-*E. coli*) found in the first 4 days after contamination. In unirradiated conventional mice, the average concentration of the various biotypes was of the same order of magnitude as the concentration in which S.R.-*E. coli* was found. In the irradiated animals, the mean concentration of the endogenous gram negative bacteria was generally higher than that of S.R.-*E. coli*. Since a constant relation

between the mean faecal concentrations of S.R.-*E. coli* in the first 4 days and at day 14 after contamination was seen in individual mice, it seems justified to draw conclusions with regard to CR from data obtained during the first days after contamination. The increased faecal concentrations of S.R.-*E. coli* and other biotypes in the irradiated animals indicate that their CR was decreased in comparison with unirradiated controls. The decreased CR following irradiation is also reflected in the mean number of 'contaminations' and 'colonizations' found during the observation period following experimental contamination of these mice with S.R.-*E. coli*. Although both groups were maintained under identical conventional conditions, the number of 'contaminations' found in the irradiated animals was considerably higher in the irradiated animals than in the unirradiated control animals. The number of 'colonizations' was also much higher in the irradiated group.

DISCUSSION

The results of these experiments indicate that the measurement of CR in individual animals is both feasible and practicable. The CR of an individual mouse can be directly expressed as the log concentration of a specific p.p. bacterial species found in the faeces 2 weeks after contamination. Since a constant relation was found between the concentration of S.R.-*E. coli* at day 14 and the concentration during the first days after contamination, apparently the concentration during the first days can be used as well. The results summarized in Figs. 2 and 3 show that low-dose contaminations with 10^3 S.R.-*E. coli* cells generally do not result in colonization of unirradiated conventional mice. It is apparent that, since the concentration can be used as a measure, the antibiotic decontaminated mice (Fig. 1) exhibit an extremely low CR, while conventional unirradiated animals have a high CR for contaminating doses up to 10^4 S.R.-*E. coli*. For higher oral doses, the CR of conventional mice decreases. Irradiated animals fall between the extremes of the antibiotic decontaminated and conventional unirradiated mice.

In the biotyping experiments (Table 1), in which the Enterobacteriaceae species colonizing the digestive tract of conventionally maintained mice were typed, great stability in the composition of the intestinal flora of conventional mice was found. In addition, it appears that our results with *E. coli* may have wider application with respect to the colonization of other Enterobacteriaceae species in conventional mice. The mean concentrations in which the various biotypes were isolated from the daily sampled faeces of the conventional unirradiated mice were in the same range as the S.R.-*E. coli* isolated when the animals had been contaminated with 10^5 cells. This could indicate that contaminations with gram negative rods from the environment via food and other sources generally do not exceed a dose of 10^5 bacteria. The increased number of 'contaminations' in the irradiated mice could indicate furthermore that a great number of contaminants from environmental sources do not 'take' in unirradiated mice. According to the results of experimental contamination with S.R.-*E. coli*, this difference in the occurrence of 'contaminations' between irradiated and unirradiated mice could be due to low dose contaminations. In that case, the dose range of contaminations from environmental

sources that result in noticeable concentrations for less than a week is apparently quite small and varies between 10^4–10^5 bacteria for unirradiated mice. The contamination dose that results in a 'contamination' is then extended in irradiated animals into the lower dose range of less than 10^4 bacteria. Our results, depicted in Figs. 1 and 3, may confirm this assumption. They indicate that irradiated animals become colonized after relatively low (10^3) oral doses of contaminating S.R.-*E. coli*. The decreased CR in irradiated mice is furthermore reflected in the increased concentrations of biotypes other than S.R.-*E. coli* as well as in the mean number of 'contaminations' and 'colonizations' (Table 1). While in the unirradiated animals the mean concentration of S.R.-*E. coli* was of the same range as that of other Enterobacteriaceae biotypes in the faeces, the concentration of S.R.-*E. coli* in irradiated mice was much lower than that of several faecal Enterobacteriaceae biotypes (Table 1). This cannot be attributed to increased contaminations from environmental sources after irradiation, because the unirradiated control animals maintained under the same environmental conditions did not show it. This 'selective' change in CR after irradiation could possibly be due to a decrease in the production of corresponding IgA antibodies in the gut. Bazin, Maldague & Heremans (1970) and Bazin *et al.* (1971) have described a considerable decrease in the number of IgA-producing cells in the intestines following lethal irradiation. The lack of sufficient intestinal antibodies preventing adherence and colonization (Freter, 1972; Williams & Gibbons, 1972) could then be responsible for the effect. Both authors conclude from their experiments that the prevention of bacterial adherence to the mucosa reduces the chance of colonization. Because surface epithelial cells are continually shed, colonization requires continuous reattachment; otherwise, the bacteria that tend to colonize are removed by mechanical factors such as secretions and peristaltic movement. To understand why some biotypes grow out in higher concentrations after irradiation than others, the following points could be of importance: first, the dose of S.R.-*E. coli* given appears to influence the concentration in which this contaminant is isolated from the faeces (i.e. colonizes the intestines) and second, different doses of different gram negative species are required to obtain the same faecal concentration (van der Waaij *et al.* 1971). The various Enterobacteriaceae species therefore will colonize the intestines in different concentrations most of the time. This is confirmed by our present quantitative biotyping experiments. Those biotypes that colonize the intestines in higher concentrations during the first days after irradiation may use up the available IgA more rapidly than those which colonize the intestines in lower concentrations. The available concentration of anti-S.R.-*E. coli* IgA could have been decreased less than that of IgA to several of the endogenously colonizing biotypes.

These findings imply that irradiated animals have a decreased CR. Such animals therefore must be carefully isolated to prevent exposure to contaminations inherent in the conventional laboratory animal environment, because once p.p. bacteria colonize, they form a potential danger for infection. Protective isolation should be considered mandatory for animals that have been decontaminated and irradiated in which the CR has decreased to extremely low values. Consequently, colonization occurs in high concentrations which implies the occurrence of spread

into lymphatic organs such as mesenteric lymph nodes and spleen (van der Waaij, de Vries & Lekkerkerk, 1972).

To measure CR in individuals such as human patients or monkeys with decreased resistance to infection, one must take advantage of the contaminations to which such individuals have been exposed in the conventional environment. Experimental oral contamination with a known dose of a particular p.p. bacterial species as in the present study is generally not possible in such individuals, as it involves a risk of infection caused by the contaminant. However, if the mechanism responsible for CR is similar in primates and mice, the measurement of CR could be accomplished by quantitative biotyping of one or more Enterobacteriaceae species isolated from faeces collected at 3 or 4 daily intervals such as has been done by Dankert (1973). The mean concentration of the typed bacteria is determined from the time related concentration curve. Typing of coliforms, klebsiella, and proteus species, as well as the determination of the concentration in which they are present in the faeces, will then give information concerning the CR of the intestinal tract to these gram negative bacteria. Determination of the concentration of the various Enterobacteriaceae species is not sufficient in itself, since different biotypes of the same species may subsequently determine the faecal concentration. Theoretically, a high CR can be misinterpreted as a low CR if the conclusion is based on the peak concentration following high dose daily contaminations with different bio- or sero-types of the same bacterial species.

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