Table 2. Spleen cells ^{3}H-thymidine incorporation in the presence of 50 μg/ml *T. cruzi* antigen, 20 μg PPD and 10 μg PHA: 1) animals vaccinated with *T. cruzi* treated with actinomycin-D; 2) normal unvaccinated animals (control); 3) vaccinated and sensitized with BCG 20 days before test; 4) sensitized with BCG only (control).

Groups (5 animals)	^{3}H-thymidine incorporation (cpm ± SD) × 10^{-3}		
	T. cruzi antigen (50 μg/ml)	PHA (10 μg/ml)	PPD (20 μg/ml)
1	7.82 ± 0.93	15.05 ± 1.50	–
2	0.92 ± 0.25	16.53 ± 1.02	–
3	–	–	3.27 ± 0.72
4	–	–	3.83 ± 0.64
P(t)[a]	< 0.001	NS[b]	NS

[a] Probability, Student's t-test. [b] No significance.

Similar results were consistently demonstrated in several lots of mice. The vaccinated mice displayed normal delayed (48 h) footpad reactions to 50 μg *T. cruzi* antigen. Vaccinated animals immunized with BCG or DNFB, and later challenged, showed normal specific ear swelling reactions to intradermal tests with 10 μg PPD and to epicutaneous tests with 0.1% DNFB solution. At the same time, these animals gave normal in vitro responses to *T. cruzi* antigen, to PPD and PHA as assessed by ^{3}H-thymidine incorporation.

Discussion

The results of the experiments presented here showed clearly that the metacyclic culture forms of *T. cruzi*, Y strain, previously treated with an adequate dose of actinomycin-D (50 μg/10^7 parasites) and injected s.c. at 7-day intervals (3×10^7 non-replicating parasites), do not induce humoral or cellular immunosuppression in adult DBA/2 mice.

Experiments carried out in our laboratory have repeatedly shown that these metacyclic forms of *T. cruzi*, Y strain, which have become incapable of multiplication by the intercalating action of actinomycin-D on the parasite DNA, represent an excellent immunogen, capable of inducing a vigorous stimulation of the immune response followed by a long lasting state of immunostimulation.

Several reasons can be invoked to explain the favorable results obtained in the vaccination with our immunogen: 1) There was no multiplication of the parasites inside the antigen-presenting cells (macrophages, dendritic cells); this made possible the presentation of *T. cruzi* antigens to T-lymphocytes under conditions much more efficient than those found in macrophages heavily loaded with replicating parasites. 2) The parasites were unable to enter nonphagocytic cells, thus the disturbing effects of a high degree of tissue parasitism were avoided. 3) The actinomycin-D-treated *T. cruzi* keep their mRNA with an apparently normal function for a relatively long period of time (about 15 days in vitro). 4) As shown here, the actinomycin-D-treated parasites can no longer induce immunosuppression, even after repeated s.c. inoculations of large numbers.

Research supported by CNPq (No. 40.0902/85).

* Author to whom correspondence should be addressed.

1 Beltz, L. A., Sztein, M. B., and Kierszenbaum, F., J. Immun. *141* (1988) 289.
2 Clinton, B. A., Ortiz-Ortiz, L., Garcia, W., Martinez, T., and Capin, R., Exp. Parasit. *37* (1975) 417.
3 Cunninghan, D. S., and Szenberg, A., Immunology *14* (1969) 599.
4 Cunninghan, D. S., Kuhn, R. E., and Rowland, E. C., Infect. Immun. *22* (1978) 155.
5 Cunninghan, D. S., and Kuhn, R. E., J. Parasit. *66* (1980) 16.
6 Kierszenbaum, F., Immunology *44* (1981) 641.
7 Maleckar, J. R., and Kierszenbaum, F., Int. J. Parasit. *14* (1984) 45.
8 Queiroz da Cruz, M., Bambirra, E. A., and Oliveira-Lima, A., Mem. Inst. Oswaldo Cruz *81* (1986) 120.
9 Ramos, C., Scadtler-Siwon, and Ortiz-Ortiz, L., J. Immun. *122* (1979) 1243.
10 Reed, S. G., Larson, C. L. L., and Speer, C. A., Z. Parasitenkd. *52* (1977) 11.
11 Scott, M. T., Immunology *44* (1981) 409.

0014-4754/89/11-12/1131-03$1.50 + 0.20/0

Lysosomal mutations increase susceptibility to anaesthetics

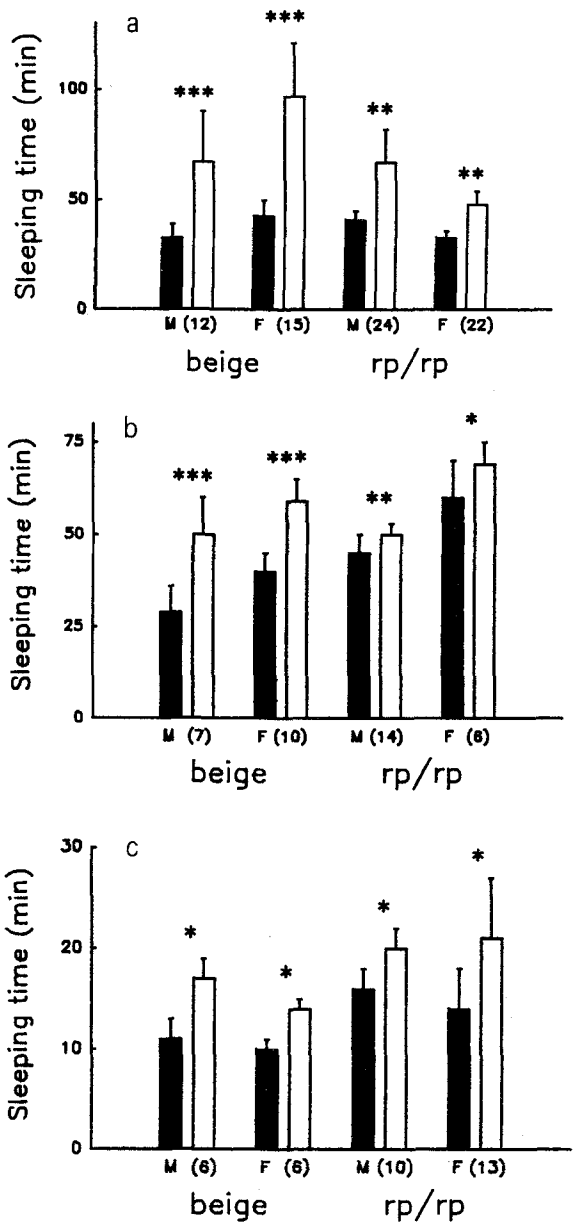
F. Ahmed, L.-G. Lundin* and J. G. M. Shire

*Department of Biology, University of Essex, Colchester CO4 3SQ (England), and *Institute for Physiological Chemistry, University Biomedical Centre, S-75123 Uppsala (Sweden)*
Received 6 March 1989; accepted 2 June 1989

Summary. The anaesthetic responses of homozygous mutant mice were compared with those of their normal heterozygous littermates. The two recessive mutations studied were beige (*bg*) and reduced pigmentation (*rp*). Homozygosity for either significantly increased the sleeping time of both sexes after treatment with pentobarbital, tribromoethanol or the steroid anaesthetic alphaxalone.
Key words. Pentobarbital; tribromoethanol; alphaxalone; sleeping time; beige mutation; reduced pigmentation gene; mice.

A wide variety of chemicals can act as general anaesthetics but their mechanism(s) of action are the subject of much discussion [1, 2]. Identification of their sites of action would be easier if there were identifiable genetic variants affecting the characteristics of individual components of the response mechanisms, whether these are lipids or proteins. Polygenic differences in susceptibility to individual anaesthetics are known in people [3] and in mice [4, 5], but seem to involve differences in the metabolic clearance of individual drugs rather than differences in the responsiveness of the anaesthetic target organs. Allelic variation at single loci appears rare, although lethal hypersensitivity to halothane in both pigs [6] and people [7] is controlled by a single allele.

In the course of an investigation [8] into the immunological characteristics of mice homozygous for the mutation reduced pigmentation (*rp*), one of the authors (L.G.L.) found that they had a high mortality when operated upon under pentobarbital anaesthesia. An initial comparison of the sleeping time induced by 100 mg/kg of pentobarbital in nine normal C57BL/6By females and seven *rprp* females suggested a difference in anaesthetic susceptibility. Sleeping time as a measure of anaesthetic potency can be affected by a variety of environmental factors such as age, diet, bedding material, temperature, time of day and exposure to inducers of hepatic metabolism [4]. Consequently the experimental design adopted was based on the simultaneous comparison of pairs of litter mates of the same sex, one homozygous for the mutation and the other heterozygous. Crosses of known heterozygotes to mutant homozygotes produced the two kinds of mice in equal proportions. As the *rp* mutation was known to affect lysosomal function [10] as well as pigmentation a second mutation was included to see whether any effects were common to lysosomal mutations or were specific to *rp*. The second mutation was beige (*bg*), a homologue of the variant causing Chediak-Higashi disease in humans [10, 11]. Three different anaesthetics (a barbiturate, a halogenated hydrocarbon and a steroid) were used to test whether susceptibility was specific to pentobarbital or reflected a general effect on anaesthetic responsiveness. The beige mutation was segregating on a C57BL/6J background and the reduced pigmentation mutation on a C57BL/10ScSn background [12]. Littermate pairs of the same sex, between six and eight weeks old, were weighed and then injected intraperitoneally with the anaesthetic under test. The dose of sodium pentobarbital (Sigma), dissolved in phosphate-buffered saline, was 45 mg/kg. Tribromoethanol (Aldrich) dissolved in t-amylalcohol and diluted with phosphate-buffered saline, known as Avertin [13], was used at 125 mg/kg. Alphaxalone (Saffan; 3 hydroxy-5 α pregnane 11, 20 dione from Glaxovet) was used at 27 mg/kg for males and 36 mg/kg for females. No mouse was anaesthetised more than once. The time for each mouse to regain its righting reflex was measured. The mean values shown were calculated from the combined values of all mice of the same sex and genotype, excluding any mice that died. The significance of differences in sleeping time between all littermate pairs of the same sex was tested with the Wilcoxon signed-rank and sign tests [14]. Both mutations significantly increased the sleeping time caused by the injection of 45 mg/kg sodium pentobarbital (fig., a). Homozygosity for the beige mutation approximately doubled sleeping times in both sexes while the effect of homozygosity for reduced pigmentation was less striking. Both mutations also significantly increased



Mean sleeping time for male (M) and female (F) mutant (open bar) and normal (filled bar) mice treated with one of three anaesthetics: *a*, pentobarbital; *b*, tribromoethanol; *c*, alphaxalone. The lines show ± 1 SE. The significance of differences in sleeping time between all littermate pairs of the same sex was tested with the Wilcoxon signed-rank and sign tests. The significance of the individual Wilcoxon tests is shown in the figure: $*p < 0.05$, $**p < 0.025$, $***p < 0.01$. The probabilities given by the sign test were similar.

the duration of the effects of standardised doses of tribromoethanol (fig., b) and alphaxalone (fig., c). Five mice, all of them females, failed to recover from pentobarbital anaesthesia; three were homozygous for *rp* and two homozygous for *bg*. One beige female did not recover from tribromoethanol anaesthesia. All mice recovered quickly from alphaxalone. The probability, by Fisher exact test, of all deaths occurring in mutants by chance alone is 0.01. The mice that died were not included in the calculation of mean sleeping times shown in the figure but were included in the statistical analyses of the differences between matched pairs.

Bleeding times were measured in male mice by the method of Novak et al.[14]. The mean value for beige homozygotes was 28.0 ± 2.2 ($n = 7$) min whilst the mean for heterozygotes was 8.1 ± 0.8 ($n = 7$) min, confirming earlier reports[15]. Bleeding time was also prolonged, but to a lesser extent, in *rp rp* mice relative to heterozygous littermates; 17.7 ± 1.3 ($n = 7$) v. 6.4 ± 1.3 ($n = 7$) min. The beige mutation has a greater effect on anaesthetic responses than the reduced pigmentation variant. This correlates with more severe effects of beige on lysosomal enzyme levels, bleeding time, natural killer cell activity in the immune system, mast cell granulation, melanin granule size and pigment distribution in the spleen[8–12]. Differences in susceptibility could be caused by differences in metabolic clearance and breakdown of the anaesthetic or by differences in the subcellular components of the anaesthetics' target cells. Since the three anaesthetics have very different chemical formulae it seems unlikely that both mutations would each pleiotropically affect several degradative pathways. Different microsomal cytochrome P450 systems are involved in the degradation of barbiturates and steroids. Microsomal P450 genes are located on chromosomes 6, 7 (near *Gpi*), 9, 15, 17 and 19 in the mouse[16]. Both mutations studied have effects on lysosomes, including the specialised melanosomes, and map to chromosome 13 (*bg*) and to the centromeric end of chromosome 7 (*rp*)[9]. The loci coding for the cytoplasmic alcohol and aldehyde dehydrogenases are on chromosomes 4 and 19. Thus it seems more likely that the lysosomal mutations cause differences in specific target cells within the nervous system. Structures altered by the mutations would change the responses of the target cells to a given level of a variety of anaesthetics. Reduced numbers of serotonin-containing granules in blood platelets are the cause of the increased bleeding time in beige mice[15]. The granules have several characteristics in common with neurosecretory granules and synaptosomes[17] and neurotransmitters such as γ-aminobutyric acid have been implicated in anaesthetic mechanisms[19]. The recent report, by Mathiasen et al.[19], of reduced effectiveness of μ-opioid receptor agonists, but not δ-opioid receptor agonists, in beige mice suggests that lysosomal mutations may be involved in systems mediating analgesia as well as anaesthesia.

The existence of defined genetic variation affecting anaesthetic sensitivity invites investigations of their mechanism of action, together with a search for mutations affecting other components of the system mediating general anaesthesia. Identification of such variants would allow the application of the powerful techniques of recombinant DNA technology.

Acknowledgment. We thank Sheila Bareham and David Spayne for their careful animal husbandry.

1 Tas, P. W. L., Kress, H. G., and Koschel, K., Proc. natl Acad. Sci. USA *84* (1987) 5972.
2 Franks, N. P., and Lieb, W. R., Nature *333* (1988) 662.
3 Kalow, W., in: Genetic Variability in Responses to Chemical Exposure, p. 15. Eds G. S. Omenn and H. V. Gelboin. Cold Spring Harbor Laboratory 1984.
4 Vessell, E. S., Lang, C. M., White, W. J., Parsanati, G. T., Hill, R. N., Clemens, T. L., Liu, D. K., and Johnson, W. D., Fedn Proc. *35* (1977) 1125.
5 Lovell, D. P., Lab. Anim. *20* (1986) 85.
6 Smith, C., and Bampton, P. R., Genet. Res. *29* (1977) 287.
7 McKusick, V. A., Mendelian Inheritance in Man, 6th edn. Johns Hopkins University Press, Baltimore 1983.
8 Orn, A., Hakansson, E. M., Gidlund, M., Ramstedt, U., Axbert, I., Wigzell, H., and Lundin, L. G., Scand. J. Immun. *15* (1982) 305.
9 Gibb, S., Hakansson, E. M., Lundin, L. G., and Shire, J. G. M., Genet. Res. *37* (1981) 95.
10 Prieur, D. J., An Updated Bibliography of the Chediak-Higashi Syndrome of Man and Animals. Washington State University, Pullman 1987.
11 Padgett, G. A., in: Spontaneous Animal Models of Human Disease, p. 256. Eds E. J. Andrews, B. C. Ward and N. H. Altman. Academic Press, New York 1979.
12 Ahmed, F., and Shire, J. G. M., J. Hered. *76* (1985) 311.
13 Cunliffe-Beamer, T. L., in: The Mouse in Biomedical Research, vol. 3, p. 407. Eds H. L. Foster, J. D. Small and J. G. Fox. Academic Press, New York 1983.
14 Siegel, S., Non-parametric Statistics for the Behavioral Sciences. McGraw-Hill, New York 1956.
15 Novak, E. K., Hui, S. W., and Swank, R. T., Blood *63* (1984) 536.
16 Nebert, D. W., Adesnik, M., Coon, C. J., Estabrook, R. W., and Waterman, M. R., DNA *6* (1987).
17 Da Prada, M., Lorez, H. P., and Richards, J. G., in: The Secretory Granule, p. 281. Eds A. M. Poisner and J. M. Trifaro. Elsevier, Amsterdam 1982.
18 Krnjevic, K., in: Molecular and Cellular Mechanisms of Anesthetics, p. 3. Eds S. H. Roth and K. W. Miller. Plenum Press, New York 1986.
19 Mathiasen, J. R., Raffa, R. B., and Vaught, J. L., Life Sci. *40* (1987) 1989.

0014-4754/89/11-12/1133-03$1.50 + 0.20/0