Table 2. Estrogen receptor in uteri of congenic mice

Strain	*H-2*	Estrogen receptor*	K_d**
C3H/HeJ	k	142	0.8
C3H.NB/J	p	161	1.9
C3H.SW/J	b	120	0.8
B10.D2/J	d	69	1.6
B10.Br/J	k	171	0.6
C57BL/10J	b	27	1.3

* ^{3}H-estradiol bound, expressed in fmoles/mg cytosol protein (pool of 10–15 uteri). ** Equilibrium dissociation constant (moles/l $\times 10^{-10}$).

relatively low estrogen receptor values (e.g., C57BL/6J, C57BL/10J, and SM/J) and some had high values (e.g., SJL/J, AKR/J, and DBA/1J) however, there was no clear-cut correlation with the *H-2* genotype. The values of the estradiol receptor in several congenic strains, i.e., which differ from each other only at the *H-2* locus but otherwise have the same genetic background, suggested an association with *H-2* since B10.D2, B10.Br, and C57BL/10J mice, which all have the same genetic background, had different levels of estradiol receptor (table 2). The K_d was in the same range of 10^{-10} moles/l for all the strains investigated.

Discussion. The total level of cytosol protein binding ^{3}H-cortisol in the palate was found to correlate with the *H-2* genotype, hence a product of a gene near or in the *H-2* locus seemed to be the glucocorticoid receptor[7]. However, the *H-2* was not the sole determinant of ^{3}H-cortisol receptor level because C3H/HeJ and CBA/J mice, both of *H-2*^{k} genotype, had different receptor levels. The findings presented here suggested an influence of *H-2* locus on the level of available binding sites for estradiol in mouse uteri. We did not measure the level of estradiol in the circulation of various strains of mice. If this level is influenced by the *H-2* locus similar to the influence on the testosterone level[5], then its possible influence on the uterine estrogen receptor could be less evident in our assay on intact animals. The individual values of estrogen receptors for each animal within the same strain were assumed to be similar; therefore uteri from 10–15 mice were pooled. The estrous cycle can influence the estrogen binding in uterus of rats[12]. However, there are controversial reports on the concentration of estrogen receptor in the uteri of rats in different periods of the cycle: increase in proestrus[13], decrease in proestrus[14] or no significant changes in the level of estrogen receptor throughout the cycle[15]. It is known that pheromones, excreted by males, cause synchronous estrus in mice[16], therefore we kept the female mice in the proximity of males for a week before the experiments.

Our results suggest an influence of the *H-2* locus on the amount of estrogen receptor in the mouse uterus. Genetic control of various proteins without a linkage with the major histocompatibility locus is well-known. For example, a genetic variation in male mouse kidney β-glucuronidase activity, not linked to the *H-2* locus, was described[17]. Further studies of mice in different periods of the estrous cycle as well as studies in individual inbred rats should shed more light on the possible genetic control of the uterine estrogen receptors.

1 This work was supported by a grant from the Margaret Duffy and Robert Cameron C. Troup Fund.
2 A.O. Vladutiu and N.R. Rose, Science *174*, 1137 (1971).
3 D. Meruelo and M. Edidin, Proc. natl. Acad. Sci. USA *72*, 2644 (1975).
4 P. Ivanyi, K. Hampl, L. Starko and M. Mickova, Nature New Biol. *238*, 280 (1972).
5 P. Ivanyi, S. Gregorova, M. Mickova, Folia biol., Praha *18*, 81 (1972).
6 K. Yamazaki, E.A. Boyse, V. Mike, H.T. Thaler, B.J. Mathieson, J. Abbott, J. Boyse, Z.A. Zayas and L. Thomas, J. exp. Med. *144*, 324 (1976).
7 A.S. Goldman, M. Katsumata, S.J. Yaffee and D.L. Gasser, Nature *265*, 643 (1977).
8 A.K. Champlin, D.L. Dorr and A.H. Gates, Biol Reprod. *8*, 491 (1973).
9 R.B. Johnson, R.M. Nakamura and R.M. Libby, Clin. Chem. *21*, 1725 (1975).
10 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
11 G. Scatchard, Ann. N.Y. Acad. Sci. *51*, 660 (1949).
12 M. Ginsburg, N.J. MacLusky, I.D. Morris and P.J. Thomas, J. Endocr. *64*, 443 (1975).
13 P. Feherty, D.M. Robertson, H.B. Waynforth and A.E. Kallie, Biochem. J. *120*, 837 (1970).
14 C. Lee and H.J. Jacobson, Endocrinology *88*, 596 (1971).
15 J.O. White, S. Thrower and L. Lim, Biochem. J. *172*, 37 (1978).
16 W.K. Whitten, J. Endocr. *13*, 399 (1956).
17 E.M. Håkansson and L. Östberg, Biochem. Genet. *14*, 910 (1976).

Production and characterization of antibody against aflatoxin M_1

W.O. Harder and F.S. Chu[1,2]

Department of Food Microbiology and Toxicology, University of Wisconsin, Madison (Wisconsin 53706, USA), 23 October 1978

Summary. Antibody against aflatoxin M_1 was obtained after immunization of rabbits with bovine serum albumin-afla M_1 oxime conjugate. The antibody has greatest binding efficiency for afla M_1, and was less efficient for afla B_1. Cross-reaction of antibody with aflatoxin Q_1, aflatoxicol, and aflatoxin B_{2a} was weak. Aflatoxin B_2, G_1, and G_2 and afla B_1-guanine adduct showed almost no cross-reaction with the antibody. The sensitivity of the binding assay for aflatoxin M_1 detection is in the range of 1–10 ng per assay. Detailed methods for the preparation of the conjugate, production of immune serum, and methods for antibody determination are described.

Aflatoxin M_1 (afla M_1) is one of the major aflatoxin B_1 (afla B_1) metabolites in mammalian systems and has been shown in cow's milk, animal serum and urine[3–5]. It is also produced by several *Aspergillus flavus* isolates but in relatively small quantities in comparison with other aflatoxins[6]. In view of the occurrence of afla M_1 in milk due to ingestion of afla B_1-contaminated feed by cattle and its carcinogenic property, afla M_1 presents a potential hazard to human health[7–9]. It is essential to have simple, sensitive and reliable methods for monitoring afla M_1 in milk as well as other dairy products. Currently, both thin layer chromatography (TLC) and high pressure liquid chromatography

(HPLC) are used for afla M_1 analysis with a lower detection limit of 0.1 ppb for fluid milk[10-13]. A more sensitive method with a detection limit of 0.03 ppb has been reported[14,15] by employing 2-dimensional TLC. Investigations on new methods for mycotoxin analysis in our laboratory have been aimed at the radioimmunoassay (RIA). In the last few years, we have successfully obtained specific antibodies for afla B_1[16], ochratoxin A[17], and T-2 toxin[18], and have developed a solid-phase RIA for afla B_1[19].

Since the molecular weights of aflatoxins are in the range of 312-320, aflatoxins are devoid of any antigenicity. The toxins also lack a reactive group for their direct coupling to a protein carrier in antibody production. However, a carboxymethyl oxime derivative of afla B_1 was made and has been used for conjugation to bovine serum albumin (BSA) and polylysine[16,20]. Specific antibody against afla B_1 was obtained from rabbits after immunization with the BSA-afla B_1 or polylysine-afla B_1 conjugates. Nevertheless, antibody produced by this approach did not cross-react with afla M_1[16,19,20], and only gave weak cross-reactions with other afla B_1 metabolites such as aflatoxicol and afla Q_1[16]. Those results suggested that in order to develop RIA for afla B_1 metabolites, the metabolite itself should be used as a hapten in the conjugation. Since afla M_1 is one of the most important afla B_1 metabolites, efforts for development of antibody against this toxin for RIA and other purposes were carried out. In this report, properties of and methods for production of this specific antibody are presented. As it will be seen from the specificity and sensitivity, the antibody will be useful in RIA for afla M_1.

Materials and methods. Aflatoxin M_1 was prepared by the method of Stubblefield et al.[21]. Aflatoxins B_1, B_2, G_1 and G_2 were prepared earlier[22], and in some cases were further purified by the preparative TLC method. Aflatoxin B_1-guanine was kindly supplied by Dr R.C. Garner (University of York, York, England). ^{3}H-afla B_1 (2.44 Ci/mmole) and ^{3}H-afla M_1 (0.30 Ci/mmole) were obtained from Moravek Biochemicals (City of Industry, California 91745). Water-soluble carbodiimide 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC) was obtained from Story Chemical Corp., Otta Chemical Division (Muskegon, Michigan). Bovine serum albumin (BSA) was a product of Sigma Chemicals (St. Louis, Missouri). Complete (Difco 3110-60-5) and incomplete (Difco 0639-60) Freund's adjuvant were purchased from Difco Laboratories (Detroit, Michigan). Albino rabbits were obtained from Klubertanz's rabbit farm (Edgerton, Wisconsin) and tested to be Pasteurella negative before use. All other chemicals were either reagent grade or chemically pure.

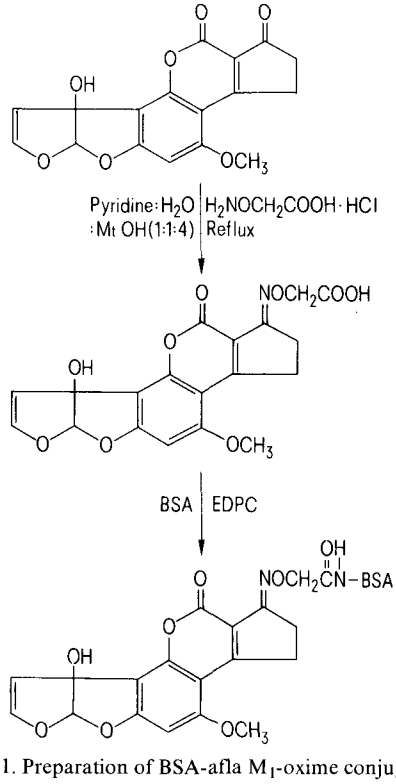

Fig. 1. Preparation of BSA-afla M_1-oxime conjugate.

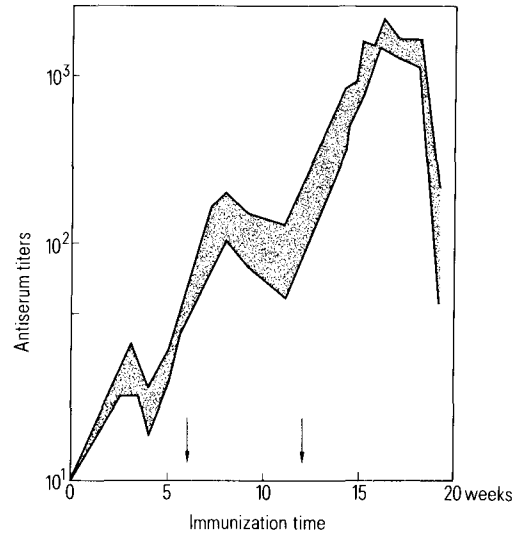

Fig. 2. Production of antibody against aflatoxin M_1. Each rabbit was immunized with 250 µg of BSA-afla M_1-oxime and given booster injections of equal concentrations (250 µg BSA-afla M_1-oxime) at times indicated by arrows. Antisera titers are expressed as the reciprocal of serum dilution required for 50% binding of ^{3}H-afla B_1. The shaded area indicates the variation of antibody titers among 3 rabbits tested.

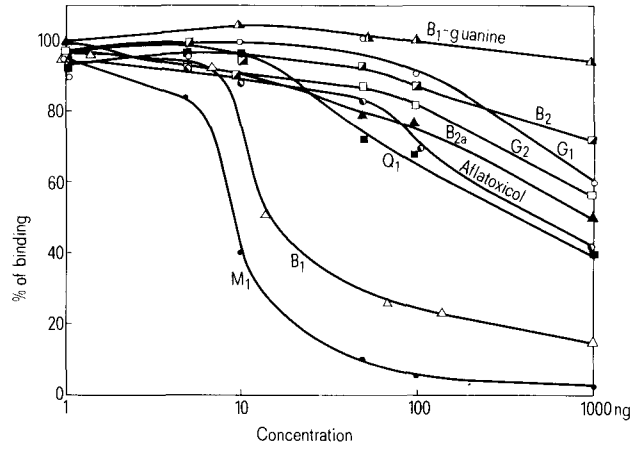

Fig. 3. Effect of different aflatoxins on the binding of ^{3}H-labelled afla B_1 with IgG. 0.1 ml of diluted antisera (diluted to give 60% binding of ^{3}H-afla B_1) was incubated with 0.1 ml of ^{3}H-afla B_1 (20-25,000 dpm) and 0.1 ml of different unlabelled aflatoxins at different concentrations at 6 °C overnight (20-24 h). All the solutions were prepared in 0.1 M NaPB at pH 7.4. The relative inhibition of binding of ^{3}H-afla B_1 with the antibody by different concentrations of unlabelled aflatoxins is shown. All values have been normalized to 100% binding.

Preparation of afla M_1-BSA conjugate. Aflatoxin M_1 was first converted to afla M_1-O-carboxymethyl-oxime (afla M_1-oxime) according to the method described by Chu et al.[23]. In the chloroform-acetone-acetic acid 50:50:10 solvent system, this preparation gives a single spot in TLC and has an R_f value = 0.43, which is different from the afla M_1 (R_f = 0.90). Afla M_1-oxime showed an absorption maximum at 350 nm with molar absorptivity of 21,475.

Antigens were prepared by conjugation of afla M_1-oxime with BSA in the presence of EDPC[16]. Details were similar to those previously described for the preparation of BSA-afla B_1-oxime conjugate[16].

Immunization schedule and antibody titer production. For immunization, the multiple-site intradermal method of Nieschlag et al.[16,24] was followed, except that the pertussis vaccine was omitted. Each rabbit received 250 µg of BSA-afla M_1 conjugate in Freund's complete adjuvant in the initial injection. Trial bleedings via the marginal ear vein were made once every week, starting 3 weeks after immunization. Booster injections were given approximately 1 week after a decrease in antibody titer was observed. The antigen was mixed with incomplete adjuvant for booster injections.

In the initial studies, binding of ^{3}H-afla B_1 and ^{3}H-afla M_1 with antisera were compared. Since the specific activity of ^{3}H-afla B_1 is much higher than ^{3}H-afla M_1, ^{3}H-afla B_1 was used as the ligand in the subsequent experiments.

Antibody titers were determined using a binding assay method[16–18]. Antiserum obtained from immunized rabbits was precipitated twice with $(NH_4)_2SO_4$ at a final saturation of 33.3%. The precipitate was redissolved in sufficient 0.01 M sodium phosphate buffer (NaPB), pH 7.4, to give a volume equal to that of the original serum sample. Appropriate dilutions (at least 3 serial dilutions) of the preparation were made for binding studies. In general, 0.1 ml of each diluted antibody solution was incubated with 0.2 ml of ^{3}H-afla B_1 in 0.01 M NaPB, pH 7.4 (9000 cpm/0.2 ml), in a cold room in complete darkness overnight. After incubation, saturated $(NH_4)_2SO_4$ was added to each dilution to a final concentration of 40%. The mixture was allowed to stand 1 hour at room temperature in complete darkness. The samples were centrifuged (5000 rpm, 10 min), and the supernatant was decanted into scintillation vials. The precipitates were washed with 0.5 ml of a 40% $(NH_4)_2SO_4$ solution, centrifuged and decanted into the same vial as the 1st decant. The radioactivity of the combined washings was counted, along with at least 4 total count samples (^{3}H-B_1, and NaPB). The degree of binding was determined. Antibody titer was defined as the reciprocal of the dilution which was required for 50% binding ^{3}H-afla B_1 under the stated conditions.

Determination of specificity of M_1 antibody. The method was essentially the same as that used for titer determination except the reaction mixture which contains: 0.1 ml of diluted serum (diluted to give 60% binding), 0.1 ml ^{3}H-afla B_1 (9000 cpm), and 0.1 ml of 0.01 M NaPB, pH 7.4, or unlabelled aflatoxins of different concentrations. The degree of displacement of radioactivity from the IgG-^{3}H-afla B_1 complex was determined.

Determination of radioactivity. For radioactivity determinations, an appropriate amount of test solution, generally less than 1 ml, was mixed with 10 ml of Aquasol (New England Nuclear, Boston, MA). Radioactivity was counted in a Beckman Model LS-330 liquid scintillation spectrometer. All the counts were corrected to dpm by a channel ratio method using known standards of tritiated toluene. The counting efficiency was generally around 20–35%.

Results and discussion. Conjugation of afla M_1 to a protein carrier can be achieved by introducing a carboxylic acid group in the molecule through either succinylation of the −OH group at the C-4 position or through the carbonyl group at the cyclopentenone ring to make a carboxymethyl oxime (figure 1). Because of limited availability of afla M_1 and of our previous experience[16], we have selected the latter approach. The results showed that approximately 5.5 moles of afla M_1 wxime were successfully coupled to 1 mole of BSA. Using the preparation as an antigen, antibody titers were demonstrated as early as 3 weeks after immunization.

In the initial study, the binding of ^{3}H-afla B_1 and ^{3}H-afla M_1 with antisera were compared. At the same protein concentration, binding of ^{3}H-afla M_1 with antisera was found almost twice as much as ^{3}H-afla B_1. However, because the specific activity of ^{3}H-afla M_1 is about 10 times less than ^{3}H-afla B_1, the apparent binding of radioactivity by the antisera was higher when ^{3}H-afla B_1 was used. Subsequently, we selected ^{3}H-afla B_1 as the ligand in all the studies. Representative results for antibody production in 3 rabbits immunized with BSA-afla M_1 oxime are shown in figure 2. It is apparent that maximum antibody production was reached 3–5 weeks after each injection, and that production was greatly stimulated by each booster injection. Highest antibody titers were obtained 15–16 weeks after the initial injection followed by 2 booster injections.

The specificity of antiserum against afla M_1 produced by rabbits after immunization with BSA-afla M_1 conjugate was determined by a competitive binding assay, and the results are shown in figure 3. Concentrations of different unlabelled aflatoxins to give 50% inhibition of binding of ^{3}H-afla B_1 were found to be 8.5, 15.0, 440, 500, and 1000 ng for M_1, B_1, Q_1, aflatoxicol, and B_{2a}, respectively. At 1000 ng tested, aflatoxins B_2, G_1, G_2 and afla B_1-guanine adduct gave less than 5% inhibition. Thus, the antiserum is most specific for binding with afla M_1 than with other aflatoxins. It is interesting to note that antibody obtained from rabbits immunized with BSA-afla B_1 did not cross-react with afla M_1. However, when the rabbit was immunized with afla M_1, the antibody did cross-react with afla B_1. Thus the hydroxyl group in afla M_1 does play a significant role in determining the antibody specificity.

From the displacement curve, it is readily seen that sensitivity for afla M_1 detection falls in the range of 1.0–10 ng. This detection range is comparable to most other chemical methods[10–12], but it is less sensitive than the RIA system for afla B_1, by which a minimum detection level of 0.1 ng per assay was reported[16,19,20]. Since ^{3}H-afla B_1 was used in all the analyses, the sensitivity could be improved considerably if a high specific activity ^{3}H-afla M_1 was available.

Present investigation indicates that antibody specifically against afla M_1 can be produced in rabbits according to the procedures reported herein, and that the specific antibody is adequate to be used in the RIA for afla M_1. Since the antibody did cross-react with afla B_1, the presence of high levels of afla B_1 together with M_1 would affect afla M_1 determination. This problem, however, could be readily resolved by the following 2 approaches: 1. Samples containing both afla B_1 and M_1 should be passed through a silica gel column according to the procedures of Stubblefield et al.[21], so that M_1 and B_1 can be separated before RIA; or 2. Afla B_1 can be determined separately by a RIA technique using antibody specifically for B_1. To increase both specificity and sensitivity of RIA for afla M_1, further studies should be directed toward sample clean-up before RIA, application of RIA for afla M_1 in biological fluids and dairy products, as well as development of an afla M_1 ligand with a higher specific radioactivity.

1 Supported by the College of Agricultural and Life Sciences, North Central Regional project NC-129, the University of Wisconsin-Madison, and by Public Health Service research grant number CA 15064 from the National Cancer Institute, NIH.

2 The authors wish to thank Dr R.C. Garner for providing aflatoxin-B-guanine adduct, and Dr Dennis H. Hseih for providing aflatoxicol and aflatoxin Q_1.
3 R. Allcroft and R.B.A. Carnaghan, Vet. Rec. *75*, 259 (1963).
4 I.F.H. Purchase and L.J. Vorster, S. Afr. med. J. *42*, 219 (1970).
5 T.C. Campbell, and J.R. Hayes, Toxic. appl. Pharmac. *35*, 199 (1976).
6 C.W. Hesseltine, O.L. Shotwell, M.L. Smith, J.J. Ellis, E. Vandegraft and G.M. Shannon, in: Toxic Microorganism-mycotoxin, botulism, p.202. Ed. M. Herzberg. UJNR Joint Panel on Toxic Microorganisms and the Dept. of Interior, Washington 1968.
7 L. Stoloff, in: Mycotoxins and other fungal related problems, p.23. Ed. J.V. Rodricks. Am. Chem. Soc., Washington 1976.
8 F. Kiermeier, G. Weiss, G. Behringer, M. Miller and K. Ranfft, Z. Lebensmittelunters. Forsch. *163*, 171 (1977).
9 F. Kiermeier, G. Weiss, G. Behringer and M. Miller, Z. Lebensmittelunters. Forsch. *163*, 268 (1977).
10 R.D. Stubblefield and G.M. Shannon, J. Ass. off. analyt. Chem. *57*, 852 (1974).
11 M. Trucksess, J. Ass. off. analyt. Chem. *59*, 722 (1976).
12 R.D. Stubblefield and O.L. Shotwell, J. Ass. off. analyt. Chem. *60*, 784 (1977).
13 L. Stoloff, J. Ass. off. analyt. Chem. *61*, 340 (1978).
14 L.G.M.Th. Tuinstra and J.M. Bronsgeest, J. Chromat. *111*, 448 (1975).
15 D.S. Patterson, E.M. Glaney and B.A. Roberts, Fd. Cosmet. Toxic. *16*, 49 (1978).
16 F.S. Chu and I. Ueno, Appl. envir. Microbiol. *33*, 1125 (1977).
17 F.S. Chu, Fred C.C. Chang, and R.D. Hinsdill, Appl. envir. Microbiol. *31*, 831 (1976).
18 F.S. Chu, S. Grossman, R.D. Wei, and C.J. Mirocha, Appl. envir. Microbiol. *37*, 104 (1979).
19 P.S. Sun and F.S. Chu, J. Food Safety *1*, 67 (1977).
20 J.J. Langone and H. Van Vunakis, J. natl Cancer Inst. *56*, 591 (1976).
21 R.D. Stubblefield, G.M. Shannon and O.L. Shotwell, J. Am. Oil Chem. Soc. *47*, 389 (1970).
22 F.S. Chu, J. Ass. off. analyt. Chem. *54*, 1304 (1971).
23 F.S. Chu, M.T. Stephen Hsia and Piera Sun, J. Ass. off. analyt. Chem. *60*, 791 (1977).
24 E. Nieschlag, H.K. Kley, and K.H. Usadel, in: Steroid Immunoassay. Ed. E.H.D. Cameron, S.G. Hillier and K. Griffiths. Alpha Omega Publishing Ltd., Wales, U.K., 1975.

Serum proteins of mice with splenomegaly[1]

H.C. Dung and Jen Yu

Department of Anatomy, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio (Texas 78284, USA), and Department of Physical Medicine and Rehabilitation, The University of Texas Healt Science Center at San Antonio, and Audie L. Murphy V.A. Hospital, San Antonio (Texas 78284, USA), 15 November 1978

Summary. Our earlier serum electrophoretic study in 'lethargic' mutant mice showed that the quantity of protein in 1 band is inversely related to the size of the spleen. In this study, we demonstrate that this protein band almost entirely disappears in mice with splenomegaly following spontaneous skin infection. The results suggest that this serum protein may play a role in regulating growth of lymphoid tissue.

In a study of serum proteins[2] from the 'lethargic' mouse, a neurological mutant[3,4], we noted that a protein band (designated as band 2) showed a quantitative change inversely associated with the growth of lymphoid organs, particularly the spleen. This protein band was quantitatively small when the spleens of the mice were in a stage of rapid growth, and increased in quantity when the spleens decreased in weight[2]. It was suggested that band 2 might contain a protein or proteins which act like a chalone to regulate lymphoid cell proliferation in the spleen.

To further substantiate our hypothesis, sera from other mice which exhibit splenomegaly have been studied electrophoretically using polyacrylamide gels (Bio-Rad Laboratories, Richmond, Cal.). The mice studied show an obvious splenomegaly associated with a skin infection. Etiology of the skin infection is not known; it occurs in approximately 25% of the phenotypically normal mice of our breeding colony over 4 months of age but is very rare in 'lethargic' animals. Mice in the colony have been brother-sister inbred for more than 13 years (over 60 generations). The infection invariably begins as a small superficial lesion over the scapular region. At this early stage, the spleens of the mice appear to be normal in size and weight. The skin lesion gradually extends to other dorsal regions of the body and erodes deeply resulting in sloughing of the fur and skin.(figure 1). Mice with the skin infection usually die in 3 months; however, some of them survive as long as 8 months after the onset of the infection.

Materials and methods. Sera were collected from 13 mice (5 males and 8 females) with normally appearing skin (control group), and 14 mice (5 males and 9 females) with skin infections (infected group) that were present from 2 to 5 weeks before blood collection. The mice were 5–7 months old at the time of sacrifice. Body and spleen weights of the mice at the time of sacrifice are summarized in table 1.

Table 1. Body and spleen weights of mice used for serum electrophoretic analysis

	Control	Infected	Statistical difference*
No. of mice	12	13	
Body weight (g)	36.4±0.3**	35.3± 1.1	NS
Spleen weight (mg)	104.7± 3.6	634 ±147.4	$p<0.001$

* Student's t-test; ** mean ± SE.

Table 2. Percentage of 11 different protein bands from mouse sera separated by polyacrylamide gel electrophoresis

	Protein band (% of total gel OD)										
	1	2	3	4	5	6	7	8	9	10	11
Control group	45.9** ± 2.6	13.1 ± 1.4	4.4 ± 0.2	14.6 ± 1.9	2.8 ± 0.3	4.3 ± 0.3	3.0 ± 0.4	2.6 ± 0.4	1.9 ± 0.3	6.0 ± 1.2	1.4 ± 0.2
Infected group	48.9 ± 5.8	5.0 ± 1.0	5.3 ± 1.1	16.3 ± 3.3	3.0 ± 0.4	4.1 ± 0.5	4.4 ± 0.7	3.6 ± 0.5	2.7 ± 0.4	5.8 ± 0.4	1.0 ± 0.2
Statistical difference*	NS	<0.001	NS	NS	NS	NS	NS	NS	NS	NS	NS

* Student's t-test; ** mean ± SE; NS = no significance.