incision and the cloaca was closed by means of a silk suture as near the rectal sphincter as possible as shown by the dotted line in Fig. 1A. The rectum was then severed just proximal to the suture. At this point a few mg of powdered sulfathiazole was dusted into the body cavity through the incision.

A sterile, pliable, rubber medicine-dropper bulb with a rolled end was cut to a length of approximately 2.5 cm and the cut end inserted into the rectum 1.5 cm (Fig. 1B). The tube was then sutured to the rectum, near the end of the inserted end, by 6 sutures. The mucosa was then attached to the surface of the skin immediately around the incision by silk sutures and dusted with sulfathiazole.

A tube as described above was inserted into the cloaca approximately 0.75 cm so as not to block the ureteral openings (Fig. 1B). The tube was then attached to the dorsal and ventral lips of the cloaca by 6 silk sutures.

Collection of Urine and Feces. To collect samples of either urine or feces or both, toy balloons of a suitable size were tied with nylon string around the rolled end of the rubber bulbs which protruded from the cloacal orifice and the artificial anus.

Discussion. Birds treated surgically in this way require virtually no post operative attention. They can move about freely in about one to two hours and may be given feed and water immediately. The authors found it desirable to wait about 3 days before placing the birds on experimental treatments in order that the diuresis observed by Hester *et al*(3) and Hart and Essex(4) would be overcome, and to insure that the sutures would hold the tubes securely in place. It is usually necessary to wash the excretion tubes with water before attaching the collection balloons to remove any urates which may collect around the tubes. In about 10% of the birds in which an artifical anus was constructed, recurring blockage of the tubes was noted, and in cases where this occurred the birds were discarded. There was no mortality related to the operation itself, and experimental periods could continue indefinitely.

By using extruded tubes the collection balloons can be attached and removed easily without the inconvenience of a harness on the bird, and contamination of the feces by the urine is prevented.

1. Davis, R. E., J. Biol. Chem., 1927, v74, 509.
2. Coulson, E. J., Hughes, J. S., Poultry Sci., 1930, v10, 53.
3. Hester, H. R., Essex, H. E., Mann, F. C., Am. J. Physiol., 1940, v128, 592.
4. Hart, W. M., Essex, H. E., ibid., 1942, v136, 657.
5. Ainsworth, L., Poultry Sci., 1965, v44, 1561.

Received July 14, 1966. P.S.E.B.M., 1966, v123.

Autoimmunity in Relation to Aging as Measured by Agar Plaque Technique.* (31502)

W. H. HILDEMANN AND ROY L. WALFORD (Introduced by A. F. Rasmussen, Jr.)
Departments of Medical Microbiology and Immunology and of Pathology, University of California School of Medicine, Los Angeles

Although alloantibody (isoantibody) titers in man tend to decline with age, striking increases in the incidence of various autoantibodies in supposedly healthy older humans are encountered(1). Not much is known in this regard with respect to old non-human populations. The present study was directed toward the possible detection of autoimmune phenomena in "normal" old mice of a strain combination in which clinically apparent hemolytic anemia and lymphomatous disease have a negligible incidence. If autoimmune phenomena contribute to chronic, perhaps subclinical, deleterious changes associated with aging(1), increased numbers of lymphoid

* Supported by research grants HD-01252 and HD-00534 from Nat. Inst. Health.

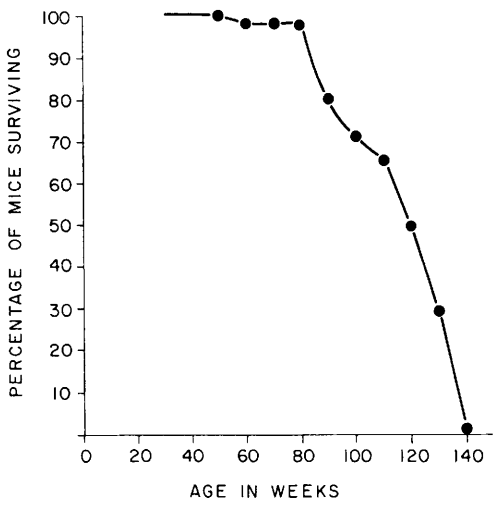

FIG. 1. Life span of 40 (C57Bl/10 x B10.LP)F1 male mice. The range of individual survival time was 60 to 140 weeks with a mean of 120 weeks.

cells secreting autoantibodies might be demonstrable in "normal" old individuals. With the recent development of a sensitive, agar plaque technique that reveals allohemolysin production by spleen cells from immunized animals *in vitro*(2), it also became possible and desirable to determine autohemolysin production as a function of age. Since a considerable number of old (C57Bl/10 × B10. LP)F1 male mice were available along with definitive life-span data on these congenic ($H\text{-}3^a/H\text{-}3^b$) hybrids, comparative autoimmune plaque assays were performed with old (30-34 mo.), middle-aged (7-12 mo.) and young adult (1.3-3 mo.) animals. The natural life span of these mice under near-optimal laboratory conditions is shown in Fig. 1. Their average life expectancy was 120 weeks or 30 months.

Materials and methods. The agar plaque technique employed(2) was essentially similar to that originally devised by Jerne *et al*(3) for measuring the responses of individual antibody-producing cells of mice to sheep red cells *in vitro*. The bottom agar layer, which serves as a base for the agar overlay containing the test cells, consisted of one per cent filtered Ionagar No. 2 (Oxoid) prepared in Hanks' balanced salt solution. DEAE dextran (Pharmacia 50 mg/100 ml) was added to neutralize the anticomplementary properties of the agar, followed by sodium bicarbonate to adjust the pH to 7. The agar to which spleen cells and autologous red cells were subsequently added was a 0.7% solution of Ionagar No. 2 also prepared in Hanks' solution. This overlay agar solution was ultracentrifuged while hot to remove fine particulate debris, a source of spurious plaques, and invariably present in all commercial agars. After discarding the pellet of debris, the agar was dissolved, cooled to about 70°C, and DEAE dextran (50 mg/100 ml) was added. After mixing, the pH was adjusted to 7.0-7.2 with 7.5% $NaHCO_3$ and the agar was distributed in 2.5 ml volumes into 1.8 × 15 cm tubes fitted with "Bacti-Capall" tops in a water bath at 47°C. To each tube of 2.5 ml agar solution at 47°C was added 0.1 ml of a washed red cell suspension (600-700 × 10^6 cells) followed by 0.2 ml of spleen cell suspension (6-18 × 10^6 cells). After mixing by rotation to avoid bubbling, the test cells were immediately poured onto the center of a previously prepared Ionagar plate (100 mm × 15 mm) and quickly dispersed over the entire surface. The cell-agar overlay suspensions at the time of pouring were at a pH of 7.0 or 7.1.

Test plates were placed in a humid 5% CO_2 incubator at 37°C for 30-35 min, followed by an additional 30 minutes in an air incubator at 37°C. About 4 ml of guinea pig complement (preabsorbed with mouse red cells at 0°C to remove naturally-occurring antibodies) at a 1:8 dilution in modified barbital buffer(4) was then added to each plate with reincubation at 37°C for 30 minutes. Excess complement was poured off and the plates allowed to incubate for an additional 2 to 3 hours at 25°C prior to final counting of the plaques. Preabsorbed, heat-inactivated (56°C for 30 minutes) guinea pig complement was used in control tests. Other control tests included plating of red cells plus buffy coat leukocytes alone in the presence of absorbed guinea pig complement. Antibiotics were not used, since spleen-derived bacteria were not found in these mice. Spleen cells were harvested with special care to assure high viability and minimal clumping. Spleen cell aggregates were disrupted in stepwise aliquots in

cold Hanks' solution in a Kontes Duall tissue grinder using minimal pestle pressure. After washing in the cold, the spleen cell suspensions were passed through a stainless steel wire mesh fitted inside a 2 ml syringe to remove any remaining debris or cell clumps. Spleen cells so harvested routinely showed 85-90% viability on the basis of trypan blue or eosin-Y exclusion. The effects of variations in technique and problems of plaque scoring are discussed in detail elsewhere(2). The most desirable innovations are prior ultracentrifugation of overlay agar to remove fine particulate debris, incubation of test cells in a CO_2 tissue-culture environment, and use of guinea pig complement from which naturally-

TABLE I. Autoimmune Hemolytic Plaque Production by Spleen Cells from Young, Middle-Aged, and Old (C57Bl/10 × B10.LP)F1 Male Mice.*

17 old mice			17 middle-aged mice			17 young mice		
Age in mo	Plaque-forming cells/10 × 10^6 viable spleen cells	Total spleen cells tested (millions)	Age in mo	Plaque-forming cells/10 × 10^6 viable spleen cells	Total spleen cells tested (millions)	Age in mo	Plaque-forming cells/10 × 10^6 viable spleen cells	Total spleen cells tested (millions)
30	.8	55	7	4.0	22	1.5	.9	66
30	.9	85	8	.8	30	1.5	1.4	84
30	1.3	50	8	1.3	36	1.5	1.9	139
30	2.8	55	8	1.7	47	1.5	3.7	105
30	6.5	31	8	3.9	77	1.5	5.0	102
30	7.7	13	8	4.2	72	1.5	6.2	87
30	21.7	16	8	6.4	100	1.5	12.6	98
31	5.8	64	9	.8	94	2	.1	91
31	6.3	78	9	2.3	70	2	1.3	102
31	25.7	16	10	.6	107	2	2.8	51
31	28.6	25	10	1.2	123	3	.1	120
31	32.6	26	11	1.3	93	3	.6	34
32	6.4	110	11	2.4	106	3	1.1	77
32	14.5	89	12	.6	42	3	1.3	64
32	20.9	51	12	.6	56	3	1.9	62
33	30.4	23	12	3.3	108	3	2.1	37
34	1.9	61	12	4.7	36	3	6.5	25
Mean:	12.6/10 × 10^6 cells (range = .8–32.6)		Mean:	2.4/10 × 10^6 cells (range = .6–6.4)		Mean:	2.9/10 × 10^6 cells (range = .1–12.6)	

* Each plaque count indicated is an average of 4 replicate plates for each spleen cell suspension obtained by disruption of the entire spleen. Control tests employing heat-inactivated (56°C for 30 min) guinea pig complement showed an average of 2.0 plaque-forming cells per 10 × 10^6 viable spleen cells (range = 0.0–7.0) in 14 young and middle-aged mice. In *t*-tests of matched-pair differences between means of plaque-forming cells for old *vs* middle-aged, old *vs* young, and middle-aged *vs* young, $P = <.005$ for the first two comparisons and $P = >.50$ for the last.

occurring mouse-specific antibodies have been absorbed.

Results. Using the above technique, autoimmune hemolytic plaque production by spleen cells from a total of 51 young, middle-aged, and old (C57Bl/10 × B10.LP)F1 male mice was determined. Each plaque count indicated in Table I is an average of 4 replicate plates for each spleen cell suspension obtained by disruption of the entire spleen. Control tests employing heat-inactivated guinea pig complement showed an average of 2.0 plaque-forming cells (PFC) per 10 × 10^6 viable spleen cells (range = 0.0-7.0) in 14 young and middle-aged mice. Among mice 1.5 to 2 months of age, a mean of 2.9 PFC per 10 × 10^6 spleen cells was detected in the presence of active complement. No increase in plaque-forming cells was found in mice 7 to 12 months of age; these showed a mean of 2.4 PFC per 10 × 10^6 spleen cells. On the other hand, many old mice, aged 30 to 34 months, demonstrated a marked increase in autoimmune spleen cells with a mean of 12.6 PFC per 10 × 10^6 spleen cells. In t-tests of matched-pair differences between means of plaque-forming cells for old *vs.* middle-aged, old *vs.* young and middle-aged *vs.* young, $P = <.005$ for the first two comparisons and $P = >0.50$ for the last. Despite the broad and overlapping range of PFC values for individual spleens determined among these 3 age groups, it is apparent that the mean increase in autoimmune PFC in old mice is highly significant in comparison with both younger age-groups of mice. However, only 8 of 17 old mice showed substantially elevated numbers of autoimmune PFC relative to younger mice, indicating that the predominantly 19 S antibodies detected by this test are not always increased with advanced age. Old mice regularly showed localized accumulations of fat in their spleen which were otherwise grossly normal with respect to size and cellularity. Milky but sterile peritoneal exudates were also common in very old mice. There was much greater individual variation in the numbers of PFC among old mice (0.8-32.6 per 10 × 10^6 cells) than in younger mice. Our initial assumption that the numbers of autoimmune PFC might increase linearly with increasing age was refuted by the similarity of results with young and middle-aged mice.

Discussion. If the appearance of autoimmune PFC is attributable to immunogenetic mutation of lymphoid cells, it is surprising that such cells appeared in significantly elevated numbers (with one exception) only in old mice. Possibly the occurrence of somatic mutations is not simply cumulative with increasing age, or perhaps the effective mutation rate increases in later life. Alternatively, mutant immunocompetent cells might be more readily repressed or selectively eliminated by mechanisms akin to "allogeneic inhibition"(5) in younger animals. Increased fragility of cells of many old animals might also be invoked. This last possibility appears unlikely in view of the finding that control plates with red cells plus buffy-coat leukocytes from old animals consistently showed only a few plaques. To what extent such plaques were caused by autoimmune circulating leukocytes or by spontaneous lysis of granulocytes (spurious plaques) is unknown. The average of about 2 plaques per 10 × 10^6 spleen cells found in control tests with heat-inactivated complement may represent the level of non-immune or spurious plaques inherent in the test. On the other hand, some of these plaques may reflect the secretion of hemolytic antibodies that are complement-independent or require only low concentrations of endogenous complement. In view of the evidence (6) that guinea pig complement is efficiently activated only by 19 S mouse antibodies, potential autohemolysins of other molecular species may not have been detected in the present experiments. Alloantibodies not active as hemolysins or directed against antigens not found on red cells would of course be missed by the present assay. Thus a consistent association between autoimmunity and aging remains a real possibility. Numbers of alloimmune plaque-forming cells at least as high as those found in old autoimmune mice occur in response to immunization with strong histocompatibility antigens. There appears to be no limitation in the weakness of histocompatibility antigens capable of evoking antibodies detectible by plaque assay(2).

Summary. A substantial proportion (47%) of apparently healthy old mice possessed significantly greater numbers of spleen cells capable of autoimmune hemolytic plaque production than did middle-aged or young adult mice of the same genotype. The average plaque counts obtained with old (C57Bl/10 × B10.LP)F1 male mice 4 to 5 times higher than those obtained with comparable younger mice. This evidence supports earlier suggestions of a meaningful association between autoimmunity and aging.

We acknowledge the expert technical assistance of William Pinkerton and Beth Teviotdale.

1. Walford, R. L., in Advances in Gerontologic Research, Academic Press, N. Y., v2, in press; Walford R. L., Exp. Gerontol., 1964, v1, 67.

2. Hildemann, W. H., Pinkerton, W., J. Exp. Med., in press.

3. Jerne, N. K., Nordin, A. A., Henry, C., in Cell-bound Antibodies, Wistar Inst. Press, Philadelphia, 1963, p109.

4. Campbell, D. H., Garvey, J. S., Cremer, N. E., Sussdorf, D. H., Methods in Immunology, Benjamin, N. Y., 1964, p246.

5. Klein, G., in Isoantigens and Cell Interactions, Wistar Inst. Press, Philadelphia, 1965, p95.

6. Winn, H. J., in Ciba Foundation Symp. on Complement, Little, Brown & Co., Boston, 1965, p133.

Received July 15, 1966. P.S.E.B.M., 1966, v123.

Inhibition of Adenosine Deaminase by a Metabolite of the Nephrotoxic Drug, Puromycin Aminonucleoside.* (31503)

N. DICKIE,† L. NORTON, R. F. DERR, C. S. ALEXANDER, AND H. T. NAGASAWA

Medical Research Laboratories, Minneapolis Veterans Hospital, and Departments of Medicine and Pharmaceutical Chemistry, University of Minnesota, Minneapolis

Administration of the aminonucleoside of puromycin (PA)‡ to the rat produces a nephrotic syndrome characterized by proteinuria, hypoalbuminemia, hypercholesterolemia, edema, and ascites(1-4). The principal necleoside metabolite in 8-hour rat urine after PA administration is the monodemethylated analog, MMPA(5). Previous studies by Cory and Suhadolnik(6) showed that the didemethylated analog of PA, APA, is a substrate for adenosine deaminase (E. C. 3.5.4.4) and that N^6-methyladenosine is an inhibitor of the enzyme. Model studies by Schaeffer *et al*(7) suggested that MMPA would be an inhibitor of adenosine deaminase. The present studies demonstrate that MMPA does indeed inhibit adenosine deaminase.

* This work was supported in part by Grant HE-04983 from USPHS.

† Present address: Food and Drug Directorate, Ottawa, Ont., Canada.

‡ Abbreviations: PA, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine; MMPA, 6-methylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine; APA, 6-amino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine.

Materials and methods. Calf intestinal mucosal adenosine deaminase, Type II, and N^6-methyladenosine were purchased from the Sigma Chemical Co.

The monodemethylated analog of PA, MMPA, was prepared biosynthetically by incubation of PA with fortified rat liver microsomes according to a modification of the procedure of Axelrod(8). PA (55.6 mg) was incubated at 25° with the microsomes from 10 g of liver together with the requisite cofactors (8) for 4 hours with stirring. The reaction was stopped by heating in a boiling water bath for 10 minutes, and the aminonucleosides isolated by ion-exchange chromatography according to Wilson *et al*(9). The aminonucleosides were separated by chromatography on Whatman 3 MM paper with n-propanol-1 N NH_4OH (75:25, v/v), corresponding to Wilson's solvent system "C"(9). The MMPA band eluted with water was repurified by thin-layer chromatography on Silica Gel HF (Brinkmann) with a chloroform-methanol-2.5 N ammonia (150:100:15, v/v), (CMN) solvent. After elution from the thin-layer