Virchows Arch [Cell Pathol] (1984) 46:135–143

Virchows Archiv B

Age-related changes of epidermal cell kinetics in the hairless mouse

Olav Hilmar Iversen and Aasa R. Schjoelberg
Institute of Pathology, University of Oslo, The National Hospital (Rikshospitalet), Oslo 1, Norway

Summary. Cell kinetic variables in normal untreated hairless mice were studied in order to observe possible age-related changes. Generally, groups of 4 male and 4 female mice were subjected to study at various ages from one to 115 weeks. The number of basal and suprabasal cells per microscopic field was observed, and after injection of tritiated thymidine the mean labelling index, the average specific activity and the mean grain count were scored. After injection of Colcemid, the average number of Colcemid-arrested mitoses was counted. With flow cytometry the fraction of cells in S and in G_2+M was also observed.

In general, both the number of suprabasal cells and the proliferative variables were significantly lower in the very young mice. They increased to slightly above normal values at about 20–22 weeks of age, and then fluctuated a little with two additional possible peaks at 40–50 and around 80 weeks, respectively, and two troughs some weeks after the peaks. However, this rhythmicity was slight and not significant. Thus the only significant age-related pattern was that very young mice have a thin epidermis and low proliferative variables. These values increase up to the age of 20 weeks, and from then on there are no obvious and significant alterations, only slight rhythmic undulations almost within normal limits.

The importance of cell kinetic changes with age for epidermal carcinogenesis is discussed in relation to these observations.

Key words: Age – Cell kinetics – Epidermis – Hairless mouse – Skin carcinogenesis

Introduction

Chronobiology is presently gaining increasing importance because of the awareness that many biological functions change with time. Rhythmic and

Offprint requests to: O.H. Iversen at the above address

continuous variations with time are observed in most biological processes, and circadian and seasonal rhythmic changes in cell proliferation and hair growth are well known in the epidermis. Form and function of the body change with age, although whether the number of epidermal cell layers and the rate of epidermal cell proliferation in both mouse and man declines or increases significantly with increasing age is unclear and reports in the literature are conflicting. (For references, see Discussion.)

The skin tumour yield after a single application of a shortacting chemical carcinogen is heavily influenced by the rate of epidermal proliferation at the time of the first contact (Iversen 1982). The importance of the state of cellular proliferation in chemical carcinogenesis is manifested, for example, in the variations in tumour yield depending on the time of the day and the point in the well known hair-cycle at which the carcinogen is applied (Clausen et al. 1984; Borum 1954). After artificial alterations in the rate of proliferation, the tumour yield varies significantly (Iversen 1982). Ebbesen (1980) recently reported that the skin of old mice is more susceptible to a single application of a chemical carcinogen than middle-aged or young skin, but Borum (1957) observed the opposite effect.

Since mice have been used extensively in epidermal carcinogenesis studies, it seems to be of interest to know whether there are significant age-related changes in epidermal thickness or cell kinetics in the undisturbed skin of hairless mice.

Materials and methods

Animals. Hairless mice (hr/hr Oslo strain) of both sexes were used. The mice were kept at a 12/12 h light/darkness rhythm (07.30–19.30 hours) with controlled temperature and humidity; they were given a standard diet and water *ad libitum*. The cages were cleaned and fresh water supplied at noon three times a week.. Because of the diurnal rhythm in epidermal cell kinetics (Clausen et al. 1979) the animals were always killed at the same time of the day, i.e. around 13.00 hours.

The mice were kept in groups of 8 to a cage. It was not always possible to obtain both male and female mice of the same age from the same litter, especially in the older age groups. During the first weeks after birth it is also wellnigh impossible to determine the sex of the baby mice. Some of the results are therefore based on measurements from mice of only one sex, or – for the very young ones – from mice of undetermined sex.

Histology and counting of cell numbers. Back skin from the animals was carefully placed on a circular frame of cardboard to obtain even stretching. Areas with scratches and wounds were carefully avoided. The skins were fixed in Bouin's solution for 4 h followed by 10% buffered formalin for 20 h, embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin. In each specimen the number of epidermal basal cells and nucleated suprabasal cells were counted in 40 microscopic fields using an eye-piece 12.5×, objective 100×. Basal cells were scored as the single layer of cells attached to the basement membrane. Nucleated cells above this layer were counted as suprabasal cells.

Cell kinetic studies. The animals (usually 4 males and 4 females) were given 0.15 mg Colcemid Ciba® in 0.5 ml saline i.p. at 09.00 hours; and 3.5 h later, at 12.30 hours, the animals were given 30 μCi H3TdR in 0.5 ml distilled water i.p. The mice were then killed by dislocation of the neck at 13.00 hours, 4 h after the Colcemid injection and 0.5 h after H3TdR injection. The skins were immediately flayed off and processed. Sections from small pieces of skin were subjected to audioradiography by dipping in Kodak NTB 2 film emulsion diluted with distilled

water 1:1, exposed for 2 weeks, developed and stained with hematoxylin. In these sections the number of basal and suprabasal cells, the number of Colcemid-arrested mitotic figures and the number of labelled cells were scored.

In 100 labelled cells the mean grain count was determined by scoring the number of grains over each nucleus. Three grains over the nucleus was the minimum requirement for accepting a cell as labelled.

From other areas of the skin samples, next to the sites where the histological specimens were taken, a piece was removed with a Castroviejo keratotome. The basal cells were then separated from the suprabasal cells (Clausen et al. 1976) by trypsin digestion and shaking, thereby producing a suspension of single cells. These isolated basal cells were fixed, treated with ribonuclease and stained with ethidium bromide and used for flow cytometric measurements (FCM) of the proportion of basal cells in each of the cell cycle phases. The DNA frequency distributions were recorded by an ICP 11 Pulse Cytophotometer (Phywe AG, Göttingen, FRG) and evaluated by a planimetric method.

Epidermis from yet another area of skin on the same animals was separated from the dermis by brief heat treatment. Samples from two animals of the same age and sex were pooled, and homogenized in 5.0 ml 0.2 N perchloric acid at +4° C and centrifuged (10,000 ×). The pellet was washed twice (2 × 2.5 ml) with ice cold 0.2 N perchloric acid and once with 4 ml ethanol/ether (1:1, v/v). DNA was hydrolyzed in 0.5 N perchloric acid at 90° C for 10 min, and duplicate aliquots (0.3 ml) determined for radioactivity in a Packard Tri-Card liquid scintillation spectrophotometer. The DNA content of the supernatant was determined by the diphenylamin method.

Table 1

Age of animals in weeks	No. of suprabasal cells per vision field		
	Males	Females	Both
1	–	–	11.0±0.5
3	–	–	12.5±1.2
4	–	–	14.9±1.3
7	21.3±0.5	18.3±0.4	14.7±0.5
9	22.0±0.6	24.0±0.8	17.0±0.7
11	18.7±0.9	–	13.9±0.7
13	25.5±0.9	28.8±0.7	20.2±0.8
17	22.7±0.8	–	16.9±0.8
18	23.8±0.6	29.4±0.1	19.7±0.6
20	31.4±1.3	–	23.3±1.3
22	26.8±3.6	–	20.0±3.6
24	23.2±0.7	–	17.2±0.7
28	24.3±1.3	23.8±0.9	17.8±1.1
32	24.9±0.5	–	18.4±0.5
37	–	22.8±1.6	16.9±1.6
40	23.4±1.6	–	17.3±1.6
45	17.4±0.9	18.1±1.3	17.8±1.1
50	15.2±0.2	18.5±1.0	16.9±1.0
55	13.1±1.1	16.9±0.4	18.7±0.8
60	20.2±0.8	18.1±0.6	19.1±0.7
65	16.6±0.6	19.8±1.1	18.2±1.0
70	18.9±3.1	11.6±0.3	15.3±2.5
75	18.0±0.3	15.2±1.4	16.6±0.9
82	–	20.6±0.9	20.6±0.9
91	–	16.1±0.9	16.1±0.9
115	–	14.7±0.8	14.7±0.8

Results

The results are shown in Table 1, 2 and 3, and in Fig. 1. Only the main results, i.e. number of suprabasal cells, labelling indices and mitotic rates are documented here. All the other detailed results (number of basal cells, Mean grain counts, Specific activity, Fraction of cells in S and in G_2) can be obtained from the authors. All values are given as mean values $\pm$ s.e.m. for groups of four mice. Relative values are used for illustration in Fig. 1. The mean of all observed values for each variable is set at 1.00.

Cell numbers

The average number of *basal cells* per microscopic field was 22.4 ± 1.0. The lowest value was seen in one-week-old animals, i.e. 19.1 ± 0.3, and the highest value was measured in 18-week-old animals at 26.0 ± 0.4. There was no systematic pattern of variation.

The average number of *suprabasal cells* (Table 1, Fig. 1) was 17.1 ± 1.3 per microscopic field, with the lowest value found in one-week-old animals, i.e. 11.0 ± 0.3; the figures increased systematically up to about 11–12 weeks,

Table 2

Age of animals in weeks	Labelling index per 40 vision fields		
	Males	Females	Both
1	–	–	18.7± 1.8
3	–	–	20.6± 1.3
4	–	–	19.3± 1.4
7	23.3± 2.7	38.5± 4.3	30.9± 3.7
9	31.3± 2.0	40.0± 5.5	35.6± 3.1
11	29.3± 4.7	–	29.3± 4.7
13	53.0± 5.7	43.3± 4.8	48.1± 3.9
17	49.8± 6.1	–	49.8± 6.1
18	53.8± 4.7	48.3± 2.8	51.0± 2.7
20	43.5± 3.3	47.3± 8.6	45.4± 4.3
22	65.8± 6.8	–	65.8± 6.8
24	51.3± 4.5	–	51.3± 4.5
28	57.5± 5.2	47.8± 2.5	49.8± 4.6
32	47.8± 2.5	–	47.8± 2.5
37	–	54.0± 6.6	54.0± 6.6
40	64.3± 6.0	–	64.3± 6.0
45	65.9± 5.4	64.0±18.0	65.0± 1.7
50	58.0±10.0	105.7±16.1	73.7±12.3
55	31.1± 3.2	65.3± 6.3	48.2±12.1
60	58.0± 6.2	52.0± 7.1	55.0± 9.0
65	37.5± 8.8	45.7± 6.3	41.6± 2.9
70	66.0±10.0	61.0± 4.2	63.5± 7.2
75	65.1± 8.8	103.5± 4.1	84.3± 7.0
82	–	50.5± 2.5	50.5± 2.5
91	–	49.0± 3.1	49.0± 3.1
115	–	38.7± 2.8	38.7± 2.8

Table 3

Age of animals in weeks	No. of Colcemid-arrested mitoses per 40 vision fields		
	Males	Females	Both
1	–	–	21.1± 3.5
3	–	–	21.3± 4.7
4	–	–	23.3± 1.1
7	29.3± 5.4	20.0± 2.7	24.6± 3.3
9	35.7± 9.9	26.3± 3.1	30.3± 4.2
11	23.8± 4.9	–	23.8± 4.9
13	35.0± 2.9	19.3± 4.9	28.3± 3.7
17	34.8± 2.4	–	34.8± 2.4
18	31.8± 1.9	34.0± 3.3	32.9± 1.8
20	45.7± 1.8	36.3± 2.9	40.3± 2.4
22	42.3± 1.5	–	42.3± 1.5
24	37.0± 5.0	–	37.0± 5.0
28	31.0± 6.4	28.3± 2.3	29.6± 3.3
32	35.0± 3.0	–	35.0± 3.0
37	–	29.8± 2.0	29.8± 2.0
40	53.3± 1.3	–	53.3± 1.3
45	56.1± 2.1	55.3±10.6	55.7± 5.3
50	14.6± 1.7	45.0± 9.8	29.8±10.7
55	31.1± 3.5	44.8± 8.4	23.3± 8.9
60	40.8± 6.9	34.3± 5.3	37.6± 3.3
65	32.0± 5.7	28.3± 5.1	30.1± 4.5
70	68.8±10.6	11.8± 1.4	40.3± 7.9
75	25.8± 6.9	10.8± 3.3	18.3± 5.1
82	–	40.7± 5.7	40.7± 5.7
91	–	15.5± 1.1	15.5± 1.1
115	–	–	26.0± 1.4

from when the numbers fluctuated only slightly and mostly remained within normal limits (see Discussion). The highest number found was in 20-week-old animals at 23.3±1.3.

The *mean labelling index* (LI) (Table 2, Fig. 1) was 48.5±8.2 labelled cells per 40 microscopic fields (comprising 896±32 basal cells). The lowest value found was seen in one-week-old animals at 18.7±1.8, and the highest value found was in 75-week-old animals at 84.3±7.0. Thus the variations were marked and showed a possibly undulating pattern (see below).

The *average specific activity* was 55.0±25.8 counts/min per ng DNA, fluctuating randomly and with large variations from 18.4±1.9 up to 108.9±54.3. Apart from some low early values, the others were within normal range.

The *fraction of cells in S* (FCM) was on average 12.0±2.7% of basal cells, varying from 6.9±0.5 in 91-week-old animals, to 19.4±6.1 in 55-week-old animals.

The *mean grain count* was on average 17.3±2.8 grains per nucleus, vary-

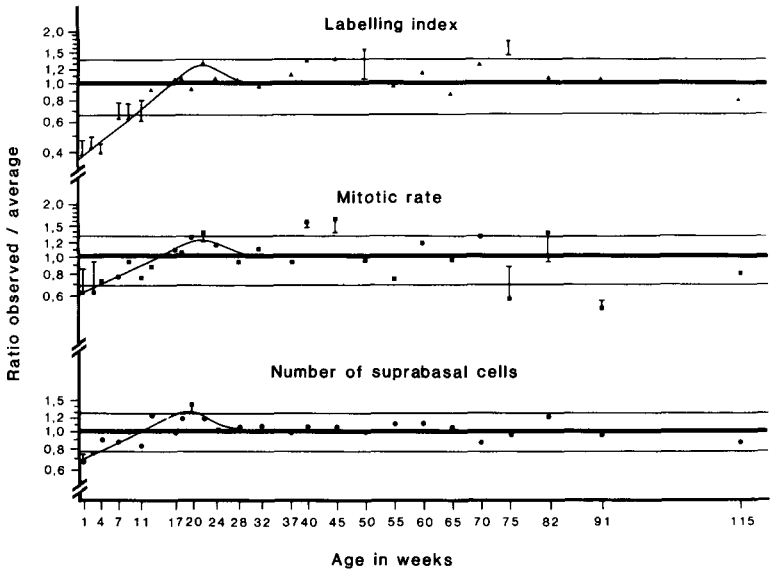

Fig. 1. The values of the three most informative variables of epidermal cell kinetics with respect to age of the animals. The mean of all observed values for each variable is set at 1.00 (the thick abscissas). The thin lines designate 95% confidence limits. For values outside the confidence limit, vertical bars indicate ± 2 s.e.m.

ing from 12.3 ± 3.1 in 60-week-old animals to 26.8 ± 5.1 in 70-week-old animals.

The *average number of Colcemid-arrested mitoses* (Table 3, Fig. 1) was 32.5 ± 5.0 mitotic figures per 40 microscopic fields, varying from 15.5 ± 1.1 in 91-week-old animals, to 40.7 ± 5.7 in 82-week-old animals.

The *fraction of cells in* $G_2 + M$ (FCM) was on average $4.3 \pm 0.7\%$ of basal cells, varying from 3.1 ± 0.7 in 65-week-old animals, to 6.8 ± 0.6 in 45-week-old animals.

In general, as illustrated in Fig. 1, the number of suprabasal cells, the DNA-related and the mitotic variables were significantly lower in very young mice, increasing to slightly above normal values at about 20–22 weeks of age, and then fluctuating a little with two possible additional peaks at 40–50 and around 80 weeks, respectively, and two troughs some weeks after the peaks.

Hence, the only significant age-related pattern is that in very young mice there were low numbers of suprabasal cells, and a low DNA synthesis and mitotic rate, which then increased to slightly above normal values at about 20–22 weeks. Following this values remained generally within normal limits (with some slight fluctuations) until the age of 115 weeks. No systematic decline in epidermal thickness or in proliferative rate could thus be confirmed in hairless mice. There were no systematic differences between male and female mice as regards epidermal thickness or cell kinetics.

Discussion

Epidermal cell proliferation in animals

Bullough (1949) studied the relationship between age and mitotic activity in the ear epidermis of the male mouse, and also observed circadian rhythms in mitotic rate. He used the colchicine technique to measure the mitotic rate, and defined the ages of the mouse as follows: immature from 1 to 3 months, mature from 3 to 12 months, middle aged from 13 to 18 months, and senile from 18 to 20 months until death. He found that in immature mice the epidermal mitotic rate was generally high, but was lowered in mature mice. In middle aged mice the mitotic rate increased again, and in senile mice it was reduced. – Bullough thus observed *slow rhythms in mitotic activity*.

Bertalanffy et al. (1965) studied the mitotic rate of rat epidermis in the growing, adult and senile rat. They measured the animals at 2, 4, 7 and 17 weeks of age, and in senile rats of more than 3 years of age. They used mainly continuous accumulation of mitoses by the Colcemid technique, and found that in the rat the epidermal *mitotic rate increased with age* to attain the maximum in adult, and particularly in senile rats. This was evident in plantar and ear epidermis, but was somewhat less marked in abdominal epidermis. He found that the increase in epidermal mitotic rate of the senile compared with the young rats amounted to approximately 50%.

Cameron (1972) measured cell proliferation in mice of various ages by the tritiated thymidine technique, injecting tritiated thymidine every 8 h for 1, 2, 3 and 4 days. He studied animals at 3, 5, 6, 8, 12 and 19 months of age and measured the labelling indices of the ear and the plantar epidermis of the hind paw. His data indicated an *age-dependent decline in the rate of cell proliferation* in all cell populations examined. He studied no animals less than 3-months-old, and hence could not draw conclusions on very young rats.

Studies in animals have thus shown varying results and have sometimes been performed with methods that would be regarded as insufficient for modern cell kinetics.

The present study shows that in young (less than 10 weeks-old) hairless mice there is a relative low number of suprabasal cells, a low mitotic rate and a low labelling index. These variables increase up to the age of 20 weeks (i.e. about 5 months), and from then on remain generally within normal limits, though with a tendency to undulations in the labelling index and the mitotic rate. In the oldest mice measured, 115 weeks (a little more than two years old), all the variables measured were within normal limits, but slightly below the average. In a previous paper Iversen and Iversen (1967) demonstrated the cycles of hair growth in hairless mice. These studies were terminated before the age of 20 weeks, and hence the undulations at 45 and 80 weeks of age cannot be correlated to any known hair growth periods.

Abortive hair growth cycles have not yet been studied in hairless mice of great age.

Hence, the present study does not confirm that the epidermal thickness or the epidermal cell proliferation rate in mice decreases systematically with increasing age. On the contrary, the rate of epidermal cell proliferation increases from birth to about 20 weeks of age, and from then on remains almost within normal limits, though there may be a slow rhythmicity.

Epidermal cell proliferation in man

There seems to be common agreement that the rate of turnover in the human epidermis *decreases with age* (Cameron 1972; Carter 1983; Grove and Kligman 1983; Kligman 1979; Marks 1981). This has been confirmed also with the tritiated thymidine technique. Groove and Kligman (1983) thus concluded that epidermal cell renewal remains relatively constant in younger subjects and then begins to fall after the age of 50 years. This confirms what was observed by Baker and Blair (1967).

Age and sensitivity to carcinogens

As regards skin carcinogenesis after a single application of a strong, complete carcinogen the general belief has been that the risk of cancer increases with age at the first exposure to the chemical carcinogen. A thorough study was published by Borum (1957). She gave a single application of 0.05 ml of 0.5% DMBA (9,10-dimethyl-1,2-benzanthracene) to the back of 388 albino mice ST/Eh. The animals were of various ages at the time of application (3, 7, 20, 42, 47, 63 and 69-weeks-old) and all had resting hair follicles. She observed that the tumour incidence was lowest, but the latent period shortest in infantile mice. In the other age groups the incidence of tumours was greater, but no significant differences were observed between these age groups. Ebbesen (1980) found the senescent mouse skin to be more susceptible to chemical carcinogens than middle-aged or young skin. Ebbesen and Kripke (1982), however, found young adult Balb/C mice more susceptible than 18-month-old animals to the induction of skin tumors by sunlamps. Using the two-stage technique (DMBA and TPA) van Duuren et al. (1975) reported a general decrease in tumour production with increasing age at the time of promotion. Also Stenbäck et al. (1981a, b) found a reduction with age in the ability of mouse skin to respond to promoters. With a short-acting single application of a chemical carcinogen (methylnitrosourea), Clausen et al. (1984) found that the highest tumour yield occurred when the carcinogen was applied at a time of low cell flux through S, G_2 and M. If cell flux through the cell cycle varies with age, this would mean that the skin cancer risk is, to a certain extent, age-dependent. In the present study we found low proliferation variables in mice less than 4-weeks-old. Hence, our results (Clausen et al. 1984, and this article) fit very well with Borum's observation (1957) that infantile mice with low cell cycle fluxes have a high epidermal sensitivity to a single application of DMBA.

In man the general risk of cancer increases with age at first exposure

(see e.g. Doll et al. 1970), but for radiation and breast cancer the opposite may be true (McGregor et al. 1977). Skin cancer in man is definitely a disease of advanced age (see e.g. Carter and Balin 1983), but this may well be due to the long latency time, and hence be only the result of long-term exposure to moderately carcinogenic doses of solar radiation and/or chemicals.

References

Baker H, Blair CP (1968) Cell replacement in the human stratum corneum in old age. Br J Derm 80:367–372

Bertalanffy FD, Pusey V, Abbott MO (1965) Mitotic rates of rat epidermis. Arch Dermatol 92:91–102

Borum K (1957) The role of the mouse hair cycle in epidermal carcinogenesis. Acta Pathol Microbiol Scand 34:542–553

Borum K (1957) Influence of age on epidermal carcinogenesis in mice. Acta Pathol Microbiol Scand 41:287–309

Bullough WS (1949) Age and mitotic activity in the male mouse, mus musculus L. J Exp Biol 26:261–286

Cameron IL (1972) Cell proliferation and renewal in aging mice. J Gerontol 27:162–172

Carter DM, Balin AK (1983) Dermatological aspects of aging. Med Clin North Am 67:531–543

Clausen OPF, Iversen OH, Thorud E (1984) Circadian variation in the susceptibility of mouse epidermis to tumour induction by methylnitrosourea. Virchows Arch [Cell Pathol] 45:325–329

Clausen OPF, Thorud E, Bjerknes R, Elgjo K (1979) Circadian rhythms in mouse epidermal basal cell proliferation. Cell Tissue Kinet 12:319–337

Doll R, Morgan LG, Speizer FE (1970) Cancers of the lung and nasal sinuses in nickel workers. Br J Cancer 24:623–632, 1970

Ebbesen P (1980) Epidermal carcinogenesis in young and aging animals. Adv Exp Med Biol 129:147–149

Ebbesen P, Kripke ML (1982) Influences of age and anatomical site on ultraviolet carcinogenesis in BALB/c mice. J Natl Cancer Inst 68:691–694

Grove GL, Kligman AM (1983) Age-associated changes in human epidermal cell renewal. J Gerontol 38:137–142

Iversen OH (1982) Enhancement of methylnitrosourea skin carcinogenesis by inhibiting cell proliferation with hydroxyurea or skin extracts. Carcinogenesis 3:881–889

Iversen U, Iversen OH (1967) Cycles of hair growth in hairless mice. Acta Pathol Microbiol Scand 69:60–62

Kligman AM (1979) Perspectives and problems in cutaneous gerontology. J Invest Dermatol 73:39–49

Marks R (1981) Measurement of biological ageing in human epidermis. Br J Dermatol 104:627–633

McGregor DH, Land CE, Choi K, Tokuoka S, Liu PI, Wakabayashi T, Beebe GW (1977) Breast cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki 1950–69. J Natl Cancer Inst 59:799–811

Stenbäck F, Peto R, Shubik P (1981a) Initiation and promotion at different ages and doses in 2,200 mice. I. Methods, and the apparent persistence of initiated cells. Br J Cancer 44:1–14

Stenbäck F, Peto R, Shubik P (1981b) Initiation and promotion at different ages and doses in 2,200 mice. II. Decrease in promotion by TPA with ageing. Br J Cancer 44:15–23

Thuringer JM, Katzberg AA (1959) The effect of age on mitosis in the human epidermis. J Invest Dermatol 33:35–39

Van Duuren BL, Sivak A, Katz C, Seidman I, Melchionne S (1975) The effect of aging and interval between primary and secondary treatment in two-stage carcinogenesis in mouse skin. Cancer Res 35:502–505

Received December 15, 1983 / Accepted January 17, 1984