MACROPHAGE–MELANOCYTE HETEROKARYONS

II. The Activation of Macrophage DNA Synthesis. Studies with Inhibitors of RNA Synthesis*

By SAIMON GORDON, M.B., Ch.B., and ZANVIL COHN, M.D.

(From The Rockefeller University, New York 10021)

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Harris and his coworkers showed that DNA and RNA synthesis could be activated in the nuclei of dormant cells after virus-mediated fusion with cells which actively make these nucleic acids (1). Subsequent studies documented the importance of nuclear swelling during the activation process, especially in relation to the increase in RNA synthesis (2).

When mouse peritoneal macrophages were fused with a mouse melanocyte cell line, the macrophage nuclei were also activated (3). These nuclei became enlarged, their RNA synthesis was stimulated, and DNA synthesis was initiated. In addition to these changes the activation of macrophage nuclei in macrophage–melanocyte heterokaryons presents a feature of special interest. Unfused macrophages do not divide in vitro because of a block which precedes DNA synthesis, in the G_0 period of the cell cycle (4, 5). It is widely held that the reentry of G_0 cells into S represents a key step in the control of DNA synthesis and subsequent mitosis in eukaryotic cells (6). We have therefore studied the initiation of macrophage DNA synthesis in heterokaryons to learn more about the G_0 state and its reversal.

In the present paper we describe the kinetics of activation of macrophage DNA synthesis in heterokaryons. The role of RNA synthesis in this process was examined using inhibitors and experiments were designed to distinguish the contributions of each parental cell to the heterokaryon.

Materials and Methods

Cell cultivation and the method of virus-induced cell fusion have been described previously (3). 2×10^5 macrophages were cultivated on 12 mm cover glasses for 1 day. 1-day old cultures of exponentially growing melanocytes were used for all the experiments reported in this paper. Freshly trypsinized melanocytes were seeded onto the macrophage monolayer for 1 hr before adding 500 hemagglutinating units of ultra-violet irradiated Sendai virus. After 30 min treatment with virus at 37°C, the preparations were washed and cultivated further in medium 199 + 10% newborn calf serum (199 M).[1]

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[1] The following abbreviations are used in this paper: MEM, Eagle's minimal essential medium; 1:1 heterokaryons, a heterokaryon containing one macrophage and one melanocyte nucleus; 199 M, Medium 199 + 10% newborn calf serum.

DNA and RNA synthesis were studied in fused preparations by radioautography. Cells were incubated in 199 M containing ^{3}H-thymidine, 10 μCi/ml for 2 hr or longer, or ^{3}H-uridine, 10 μCi/ml for 20 or 60 min. These preparations were processed as described previously (3). At least 25 1:1 heterokaryons (heterokaryons containing one macrophage and one melanocyte nucleus) were evaluated per cover glass, usually in duplicate preparations. DNA synthesis was scored + or − for each nucleus based on heavy labeling over background. RNA synthesis was evaluated by counting grains. Unfused macrophages and melanocytes served as controls in each preparation.

Chick red cell–melanocyte heterokaryons were prepared as follows: The red cells of 11 or 12-day old chick embryos were washed twice in 199 M, resuspended in 199 M, and pipetted onto glass cover slips (1×10^6/cm^2). After 1 hr at room temperature, 1×10^4 exponentially growing melanocytes, suspended in 199 M, were added to each cover slip. After another hour of incubation at 37°C the cells were treated with 500 hemagglutinating units of inactivated Sendai virus for 30 min, washed twice, and cultivated in 199M. DNA synthesis of heterokaryons was measured by radioautography, using four consecutive 2 hr pulses with ^{3}H-thymidine 1 hr after treatment with virus, and another pulse 18–20 hr after fusion.

The inhibitors used in the present experiments, actinomycin D and bromotubercidin, were obtained from Dr. E. Reich of The Rockefeller University. Concentrated stock solutions were stored at −20°C and thawed and diluted immediately before use.

The effect of these drugs on DNA, RNA and protein synthesis in unfused cells was studied by measuring the incorporation of tritiated precursors into trichloroacetic acid (TCA)-insoluble products. L-leucine-4, 5^{3}H, 44 Ci/mM, uridine-5-^{3}H, 25.9 Ci/mM, and thymidine-methyl-^{3}H, 2.0 Ci/mM, were all purchased from the New England Nuclear Corp., Bedford, Mass. and used at 5 μCi/ml in Eagle's minimal essential medium (MEM) with 10% calf serum. Replicate cultures were prepared for these incorporation studies by cultivating 1.2×10^5 melanocytes or 3×10^6 macrophages in 35 mm tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) for 1 day in MEM + 10% calf serum. The cells were washed and treated with each drug in the presence of the appropriate precursor. After drug treatments of varying duration the cells were washed 3 times with MEM and incubated further in the presence of radioactive tracer. At various times the incorporation into TCA-insoluble products was measured as follows. The cells were washed 2 times with ice-cold saline, scraped in saline, and precipitated with an equal volume of 10% TCA. The precipitates were washed 2 times in 2.5% TCA and dissolved in *N*-NaOH. Samples were counted in Bray's solution in a Mark II liquid scintillation counter with an efficiency of 34%. The results were expressed as cpm/μg protein. The protein content of samples was determined by the Lowry method (7).

Inhibitors were also used in cell fusion experiments, either preceding or following treatment with virus. In pretreatment experiments one cell partner was treated with a particular drug, washed 3 times, and fused with untreated cells 1 hr later. Controls for these experiments always included pretreatment of the other parent, as well as cells fused without drug treatment. In posttreatment experiments, the inhibitor was added to cocultivated cells either at the same time as the virus or afterwards. After treatment with the drug the fused cells were washed 3 times and cultivated further in 199M.

RESULTS

The morphological changes which take place in macrophage–melanocyte heterokaryons have been described previously (3). The macrophage nucleus becomes larger, contains more prominent nucleoli, and exhibits stimulated RNA and new DNA synthesis. The kinetics of activating macrophage DNA synthesis in heterokaryons were next determined.

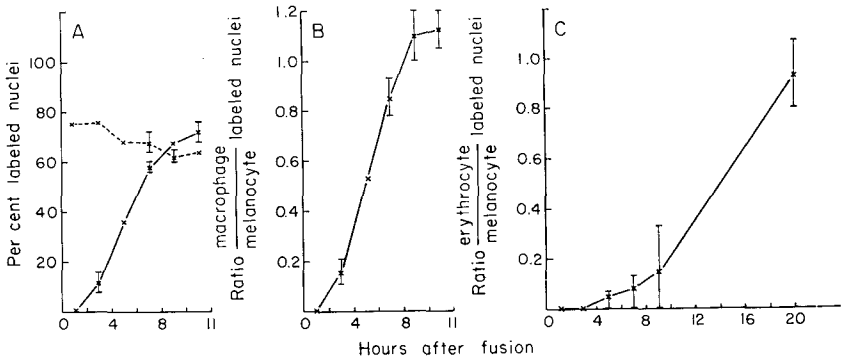


FIG. 1. DNA synthesis in 1:1 heterokaryons at different times after fusion. *a*, *b*, macrophage–melanocyte heterokaryons (X------X melanocyte labeling, X——X macrophage labeling); *c*, erythrocyte–melanocyte heterokaryons.

TABLE I

The Pattern of Labeling in Multiple Heterokaryons after a ^{3}H-Thymidine Pulse 9–12 Hr After Fusion

Cell composition*	Total No. of cells scored	Labeling pattern‡		No. of cells	Per cent of all melanocytes labeled
		Macrophage	Melanocyte		
0:1	100		+	66	66
			−	34	
1:1	60	+	+	30	57
		−	+	4	
		+	−	7	
		−	−	19	
2:1	30	++	+	14	57
		+−	+	2	
		−−	+	1	
		++	−	5	
		−−	−	8	
3:1	30	+++	+	16	60
		++−	+	2	
		+−−	−	1	
		++−	−	1	
		+++	−	3	
		−−−	−	7	
4:1	25	++++	+	12	48
		++++	−	1	
		−−−−	−	12	

* The number of macrophage nuclei is listed first, i.e., a 3:1 cell has three macrophage nuclei and one melanocyte nucleus.

‡ The macrophage nuclei are enumerated first, the melanocyte nucleus last. For example, ++−+, a 3:1 heterokaryon with two macrophage and one melanocyte nucleus labeled.

DNA Synthesis in Macrophage–Melanocyte Heterokaryons.—Newly formed heterokaryons were exposed to consecutive 2 hr pulses with ^{3}H-thymidine. DNA synthesis, at different times after fusion, is illustrated in Figs. 1 and 2. As can be seen in Fig. 1 *a*, the number of melanocyte nuclei which were labeled in heterokaryons remained constant throughout the 11 hr period after the start of fusion. The macrophage nuclei in heterokaryons, however, did not start DNA synthesis until 3 hr after fusion. Over the following 4 hr period macrophage nuclei became labeled in most heterokaryons containing a labeled melanocyte nucleus. After 9 hr about 10% of macrophage nuclei were labeled in the absence of melanocyte labeling. Melanocyte DNA synthesis in these heterokaryons was probably complete by this time.

The kinetics of macrophage DNA synthesis initiation proved to be remarkably constant. An irreducible delay of 2–3 hr and the steep rise of the activation curve were characteristic features. Since 50–80% of macrophage nuclei invariably started DNA synthesis within 3–8 hr of fusion, it was possible to assay this parameter of macrophage nuclear activation reliably. The ratio of macrophage- to melanocyte-labeled nuclei also provided a good indicator of the degree of activation, as illustrated in Fig. 1 *b*.

The activation of DNA synthesis is specific for heterokaryons. Unfused macrophages or macrophage homokaryons were never found labeled, thus excluding a nonspecific effect of cell fusion itself. Since the macrophage is capable of RNA and protein synthesis it was thought possible that the macrophage could actively inhibit melanocyte DNA synthesis in heterokaryons. This is, however, not the case, as shown in Table I. The frequency of melanocyte DNA synthesis was unaffected in heterokaryons containing as many as four macrophage nuclei. Moreover, the nuclei of multiple heterokaryons often labeled in synchrony, as illustrated also in Fig. 2 *b*.

As reported previously, more than 80% of 1:1 heterokaryons could enter mitosis within 24–48 hr of fusion (3). Further proliferation, however, was sluggish compared with that in unfused melanocytes.

DNA Synthesis in Chick Erythrocyte–Melanocyte Heterokaryons.—The above studies suggested that macrophage nuclei were induced to make DNA sooner than were chick erythrocyte nuclei placed in HeLa cytoplasm (8). DNA synthesis in macrophage and erythrocyte nuclei was therefore compared in similar cytoplasm by fusing both types of cell with melanocytes. The pooled results of three erythrocyte–melanocyte fusion experiments are shown in Fig. 1 *c*. The crosses represent the mean of three observations; the bars represent their range.

DNA synthesis in the chick nuclei started after a lag which varied from 5 hr after fusion for a few nuclei, to more than 9 hr after fusion for most of the nuclei. This heterogeneity was present among different cells within the same experiment, as well as in different experiments. By 20 hr 48–70% of red cell

nuclei were labeled. In parallel experiments macrophage DNA synthesis had started by 3 hr and was found in 70% of 1:1 heterokaryons by 7 hr. 60–70% of melanocyte nuclei were labeled in both types of heterokaryon throughout these experiments.

The macrophage nucleus therefore differs from the erythrocyte nucleus in the relative speed and homogeneity with which DNA synthesis is activated in heterokaryons.

RNA Synthesis in Heterokaryons.—Heterokaryon formation results in an increase in macrophage RNA synthesis, as reported previously (3). The kinetics of this process are shown in Table II. Stimulated RNA synthesis can be detected within an hour of fusion and shows a 2-fold increase by 2 hr (Fig. 2 *c*).

TABLE II

*The Incorporation of Uridine into Macrophage Nuclei**

Hr after fusion	Unfused cells	1:1 Heterokaryons	Binucleate homokaryons (per nucleus)		Ratio of heterokaryons to unfused cells
	Mean SD	*Mean SD*	*Mean*	*SD*	
1	22.2 ± 5.9	33.7 ± 9.8	29.5	±9.0	1.5
2	18.2 ± 4.8	33.8 ± 8.6	25.8	±7.5	1.9
3	19.8 ± 5.8	43.7 ± 10.5	26.1	±10.4	2.2
4½	19.2 ± 5.7	43.4 ± 7.7	24.6	±5.6	2.3
5½	27.4 ± 5.7	55.5 ± 16.8	32.1	±9.8	2.0

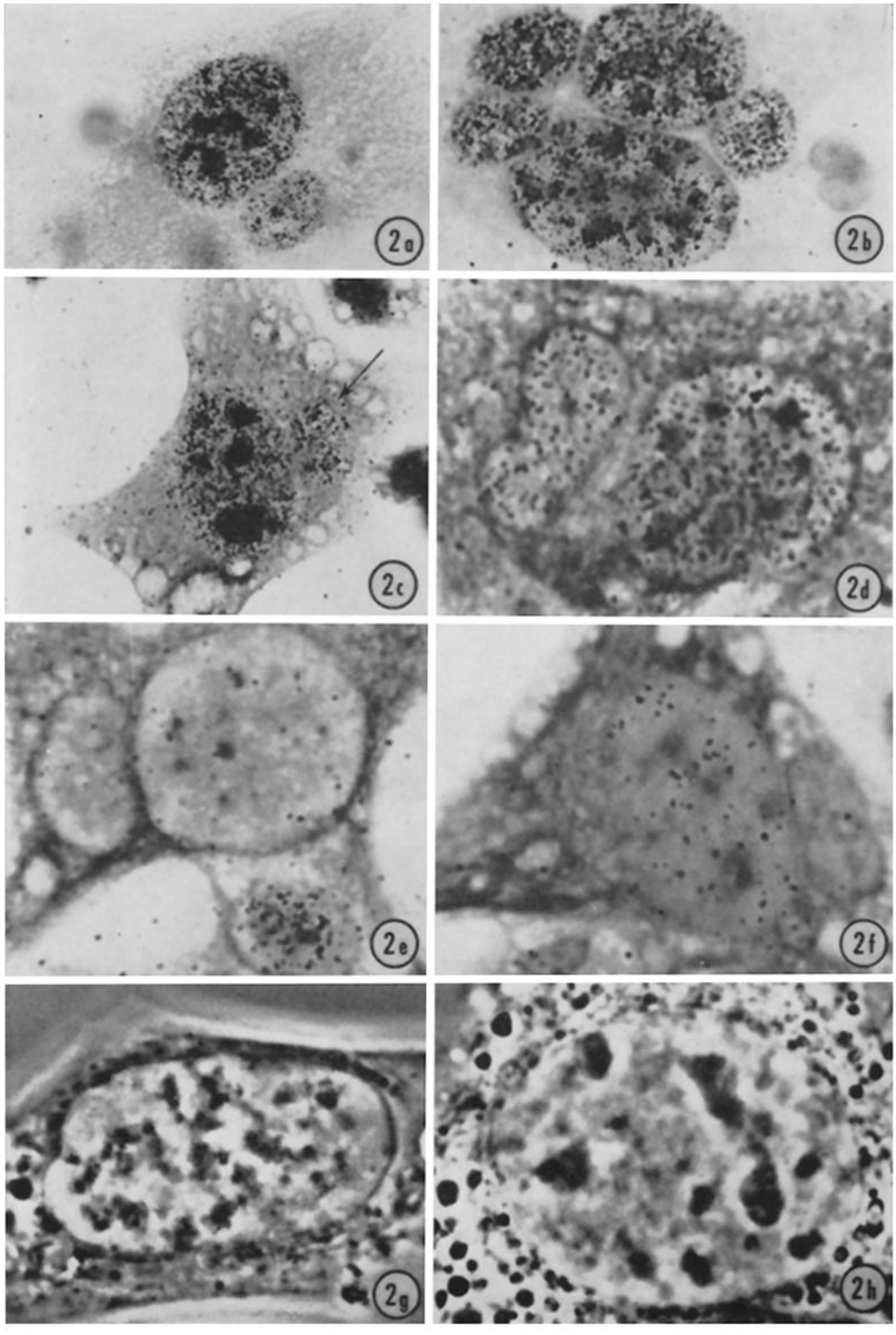
No. of grains per macrophage nucleus.

* No. of cells, 25; pulse, 20 min.

There is little further increase in macrophage RNA synthesis in heterokaryons relative to that in unfused macrophages. A lesser stimulation of RNA synthesis takes place in macrophage homokaryons.

After labeling with uridine for 20 min, grains were found over nucleoli as well as scattered over the rest of the nucleus (Fig. 2 *c*). Fewer than 10% of the total grains were present over the cytoplasm of cells. After longer periods of labeling, or after a 40 min "chase" with nonradioactive uridine, there was an increase in cytoplasmic label of both fused and unfused cells. It was not possible to establish by means of grain counts, however, whether the macrophage nuclei contributed RNA to the heterokaryon cytoplasm.

Macrophage nuclei, therefore, make RNA at the time of fusion and the further stimulation in RNA synthesis in the heterokaryons precedes their DNA synthesis. Further experiments were undertaken to determine if macrophage DNA synthesis depended on heterokaryon RNA synthesis, and in particular, macrophage RNA synthesis. Two compounds, actinomycin D and bromotubercidin, were used to inhibit RNA synthesis.


2a
2b
2c
2d
2e
2f
2g
2h

The Effect of Actinomycin on RNA, DNA, and Protein Synthesis.—Actinomycin depressed RNA synthesis irreversibly in both melanocytes and macrophages after treatment for 1 hr (Table III). 85–99% inhibition of RNA synthesis could be obtained, in both cells, in the dose range 1–5 μg/ml. When macrophages were exposed to actinomycin and ^{3}H-uridine at the same time, the inhibition of uridine incorporation occurred somewhat more slowly than in melanocytes, but after a few hours macrophage RNA synthesis was affected more extensively. Many macrophages were dead 5 hr after treatment with 5 μg/ml.

Protein synthesis in both cell types was inhibited to a lesser extent than RNA synthesis, especially in the first hours of exposure and only one-third of protein synthesis was inhibited over the 6 hr period which followed treatment with 5 μg/ml. Melanocyte DNA synthesis was unaffected for the first hour, but up to two-thirds inhibition followed treatment with 5 μg/ml.

Macrophage DNA Synthesis in Heterokaryons Treated with Actinomycin.—The results of an experiment in which heterokaryons were treated with actinomycin, ½–1 ½ hr after fusion, are illustrated in Fig. 3. Macrophage DNA synthesis, measured by radioautography, was prevented by 5 μg/ml and in-

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FIG. 2 *a*. DNA synthesis by both nuclei of a 1:1 heterokaryon. Thymidine pulse 5–7 hr after fusion. Stained radioautograph. × 1000.

FIG. 2 *b*. Synchronous DNA synthesis by three macrophage and two melanocyte nuclei in a heterokaryon. Thymidine pulse 5–7 hr after fusion. Note the absence of grains over the unfused macrophage nucleus. Stained radioautograph. × 1000.

FIG. 2 *c*. RNA synthesis in a 1:1 heterokaryon 2 hr after fusion. The macrophage nucleus (arrow) inside the heterokaryon has more grains and is larger than the adjacent unfused macrophage. Uridine pulse 20 min. Stained radioautograph. × 1000.

FIG. 2 *d*. RNA synthesis in a 1:1 heterokaryon 2 hr after fusion. The macrophage was treated with 1 μg/ml of actinomycin for 1 hr before fusion. The macrophage nucleus is enlarged and is heavily labeled. Uridine pulse 20′. Stained radioautograph. × 1400.

FIG. 2 *e*. RNA synthesis in a 1:1 heterokaryon 2 hr after fusion. The melanocyte was treated with 5 μg/ml actinomycin for 1 hr before fusion. The melanocyte nucleoli are small and round. Neither the melanocyte nor the macrophage nucleus is labeled, whereas an unfused macrophage nearby is heavily labeled. Uridine pulse 60′. Stained radioautograph. × 1400.

FIG. 2 *f*. RNA synthesis in a 1:1 heterokaryon 2 hr after fusion. The macrophage was treated with 5 μg/ml of actinomycin for 1 hr before fusion. The macrophage nucleus is virtually unlabeled. The melanocyte nucleus shows reduced but definite labeling and the nucleoli are smaller than usual, due to actinomycin cross-toxicity. Uridine pulse 60′. Stained radioautograph. × 1200.

FIG. 2 *g*. Melanocyte after 1 hr of treatment with 5 μg/ml of bromotubercidin. The nucleoli appear as numerous, discrete, small bodies. Fixed in 1.25% glutaraldehyde. Phase contrast. × 1500.

FIG. 2 *h*. Melanocyte 3 hr after washout, after a 4 hr treatment with 5 μg/ml of bromotubercidin. The nucleolar masses are larger than during treatment. Fixed in 1.25% glutaraldehyde. Phase contrast. × 1500.

TABLE III

*The Effect of Actinomycin on RNA, Protein, and DNA Synthesis in Unfused Cells**

Tracer	Dose Actinomycin	Time	Incorporation (cpm/μg protein)		Per cent Inhibition	
			Melanocyte	Macrophage	Melanocyte	Macrophage
	μg/ml	*(hr)*				
^{3}H-Uridine	0	1	150	150		
		3	508	462		
		6	948	908		
	0.1	1	74	90	51	40
		3	200	171	60	63
		6	422	235	51	74
	1.0	1	16	30	89	80
		3	63	50	88	89
		6	145	41	85	96
	5.0	1	7	21	95	85
		3	7	17	99	97
		6	25	17	97	98
^{3}H-Leucine	0	1	32	11		
		3	97	31		
		6	188	52		
	0.1	1	N.D.	11	N.D.	0
		3	N.D.	32	N.D.	0
		6	N.D.	42	N.D.	19
	1.0	1	32	10	0	8
		3	84	24	13	22
		6	171	39	9	25
	5.0	1	34	10	0	8
		3	64	22	34	28
		6	118	33	37	36
^{3}H-Thymidine	0	1	82			
		4	334			
		7	550			
	0.1	1	94		0	
		4	370		0	
		7	680		0	
	1.0	1	84		0	
		4	204		39	
		7	326		41	
	5.0	1	72		12	
		4	124		63	
		7	184		67	

* Cells were treated with actinomycin for 1 hr in the presence of radioactive precursor, washed 3 times, and incubated further in fresh precursor.

hibited, to a lesser extent, by 0.5 μg/ml. A dose of 0.05 μg/ml had no effect. The number of labeled melanocyte nuclei did not fall appreciably, even after 5 μg/ml, but the intensity of their labeling did diminish towards the end of the experiment.

Several morphologic changes were observed in preparations treated with actinomycin. The melanocyte nucleoli were characteristically small and round (see Fig. 2 *f*). There was considerable macrophage cell death after treatment with 5 μg/ml, but heterokaryons survived, like the unfused melanocytes. The swelling of the macrophage nuclei in these heterokaryons was unaffected by actinomycin treatment.

The initiation of macrophage DNA synthesis was therefore sensitive to actinomycin treatment started ½ hr after fusion. The relationship between

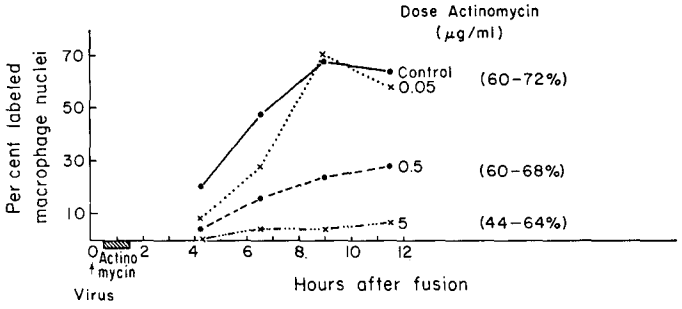

FIG. 3. The effect of actinomycin treatment on the initiation of macrophage DNA synthesis in 1:1 heterokaryons. Per cent of labeled melanocyte nuclei in brackets.

the timing of cell fusion and the period of actinomycin sensitivity was examined by treating cocultivated cells with actinomycin at different times before or after fusion.

Freshly trypsinized melanocytes were seeded on macrophage monolayers and the cells cocultivated for 3 hr before fusion. At hourly intervals, starting 2 hr before fusion and ending 5 hr after fusion, groups of cover slips were treated with 5 μg/ml actinomycin. After an hour's treatment the preparations were washed 3 times and cultivated further. All groups were exposed to ^{3}H-thymidine for two periods, 5–7 and 7–21 hr after fusion, and processed for radioautography. The results of this experiment are shown in Fig. 4.

Macrophage DNA synthesis was most severely depressed when the cells were treated with actinomycin 2 hr before fusion, but the effect was still striking when actinomycin and viral treatment coincided. Macrophage DNA synthesis was affected to a lesser degree when drug treatment was delayed till 1–3 hr after fusion, but by the 4th hr treatment was without effect. 50–60% of melanocyte nuclei were labeled in all groups. Cell fusion itself was unaffected by actinomycin treatment.

Low doses of actinomycin D have been reported to inhibit mainly ribosomal RNA synthesis (9). The selective effects of smaller doses of actinomycin on macrophage DNA synthesis and on melanocyte RNA synthesis were therefore compared. This comparison rests on the assumption, to be substantiated later, that the melanocyte provides all the RNA necessary for macrophage DNA synthesis to be achieved.

Macrophage DNA synthesis was measured by radioautography, after treating cocultivated cells with actinomycin for 1 hr before fusion and exposing the preparations to ^{3}H-thymidine 1–7 hr after fusion. RNA synthesis was measured in unfused melanocytes by treating cells for 1 hr, washing them, and measuring the incorporation of ^{3}H-uridine into TCA-insoluble product over a 4 hr period. The melanocyte nucleoli provided a sensitive morphologic indicator of actinomycin action in both types of preparation.

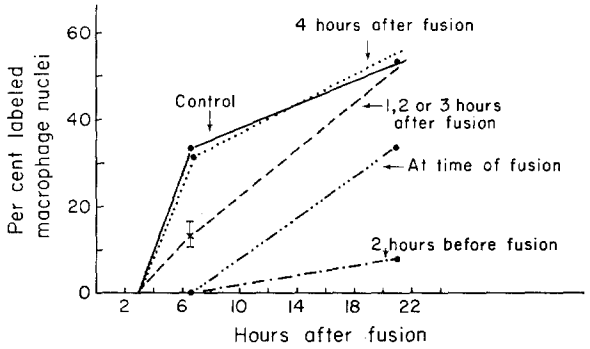

FIG. 4. Macrophage DNA synthesis in 1:1 heterokaryons after 5 μg/ml of actinomycin treatment before or after fusion.

As can be seen in Fig. 5, macrophage DNA synthesis in heterokaryons was unaffected by less than 0.1 μg of actinomycin per ml, a dose which inhibited 56% of melanocyte RNA synthesis and which brought about the characteristic nucleolar changes. At a higher dose, both macrophage DNA and RNA synthesis were progressively inhibited. The number of labeled melanocyte nuclei in heterokaryons varied between 63 and 83% and was undiminished by treatment.

This experiment showed that macrophage DNA synthesis could proceed independently of a large proportion of total RNA synthesis, presumably mainly ribosomal RNA, but depended on a species of RNA inhibited by >0.1 μg/ml actinomycin.

Selective Inhibition of RNA Synthesis before Fusion.—To answer the question whether one or both nuclei of a heterokaryon contribute RNA necessary for macrophage DNA synthesis, each cell was treated with an inhibitor of RNA synthesis before fusion with its untreated partner. The diffusion of drug in such experiments from the treated nucleus of a heterokaryon to the untreated

nucleus will be referred to as cross-toxicity. The extent to which this occurred in pretreatment experiments was evaluated from nucleolar morphology and by radioautography.

(*a*) *Actinomycin treatment before cell fusion:* Melanocytes or macrophages were treated with actinomycin, 1, 5, or 10 μg/ml, for 1 hr before fusion. The actinomycin pretreatment experiments made it possible to dissociate DNA and RNA synthesis in the macrophage nucleus of heterokaryons. After macrophage pretreatment (5 μg/ml) RNA synthesis was reduced, before and after fusion, while DNA synthesis was unaffected (Fig. 6). Macrophage RNA synthesis amounted to less than 20% of that found in untreated heterokaryons

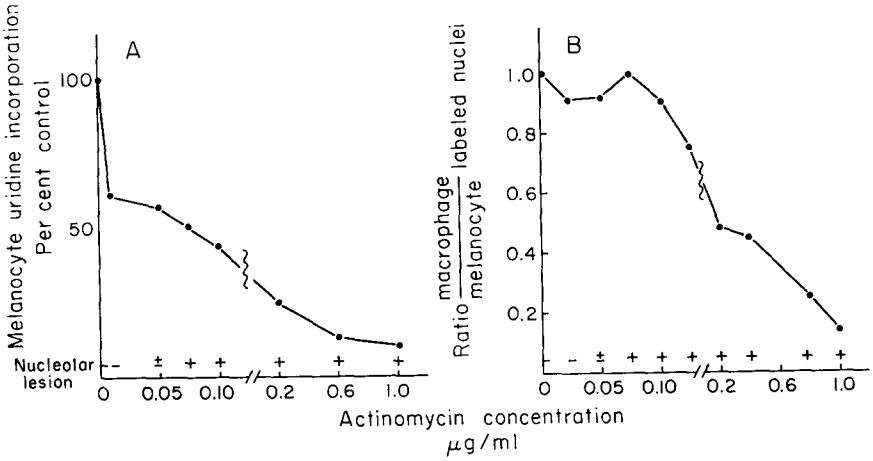

FIG. 5. The effect of different doses of actinomycin on (*a*) RNA synthesis in unfused melanocytes and (*b*) the activation of macrophage DNA synthesis in 1:1 heterokaryons.

during the 1st 4 hr after fusion. Cross-toxicity depressed melanocyte RNA synthesis in these heterokaryons only moderately (50–70% of control grain counts), as illustrated in Fig. 2 *f*. These experiments suggested that macrophage RNA synthesis was not important for its own DNA synthesis.

When melanocytes were pretreated (1 μg/ml) the macrophage nuclei made no DNA after fusion. Severe cross-toxicity abolished macrophage as well as melanocyte RNA synthesis (Fig. 2 *e*), however, so that melanocyte pretreatment did not achieve a selective effect on the melanocyte nucleus.

Cross-toxicity from the pretreated macrophage nucleus to the melanocyte nucleus of a heterokaryon became more severe (20–30% of control grain counts) after pretreating macrophages with 10 μg/ml actinomycin and was then associated with some depression of macrophage DNA synthesis. When macrophages were pretreated with 1 μg/ml actinomycin the macrophages made no RNA before fusion, but were stimulated after fusion to make as

much RNA as in untreated heterokaryons (Fig. 2 *d*) showing that the susceptibility of the macrophage nucleus to actinomycin had changed after fusion.

(*b*) *Bromotubercidin treatment of cells:* Bromotubercidin was used to distinguish between melanocyte and heterokaryon RNA synthesis. This compound is an adenosine analogue which is incorporated into RNA and reversibly depresses RNA synthesis.[2] It was therefore likely that melanocyte pretreatment with bromotubercidin would not subsequently affect macrophage RNA synthesis in heterokaryons.

(*1*) *The effects of bromotubercidin on unfused cells:* Bromotubercidin depressed 84% of melanocyte RNA synthesis (Table IV). RNA synthesis recovered

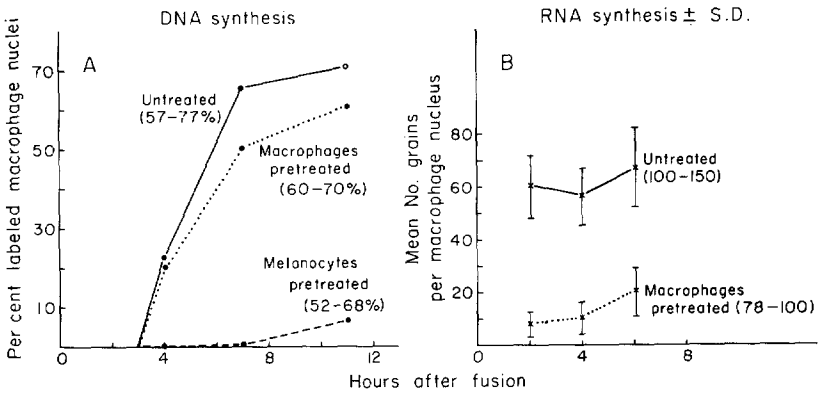

FIG. 6. Macrophage DNA and RNA synthesis in 1:1 heterokaryons after treating either melanocytes or macrophages with 5 μg/ml of actinomycin 1 hr before fusion. Melanocyte abel is given in brackets.

rapidly after 1 hr of treatment and somewhat more gradually after 5 or 9 hr of treatment. After a 6 hr period of treatment melanocyte RNA synthesis recovered rapidly over the next 8 hr from 19 to 56% of control total RNA synthesis. Protein and DNA synthesis were relatively unaffected early, but declined later from 68 to 55%, and from 75 to 54% of control, respectively. Bromotubercidin treatment caused a characteristic fragmentation of melanocyte nucleoli which was fully reversible (Fig. 2 *g*, *h*).

After 4 hr of treatment macrophages became rounded up, making 12% of the control RNA. When bromotubercidin was removed the macrophages became well spread again and RNA synthesis proceeded at the same rate as in untreated cells. Protein synthesis was little affected by these conditions (68–75% of control).

These experiments showed that bromotubercidin could suppress RNA syn-

[2] Reich, E., and B. Brdar. Personal communication.

TABLE IV

The Effect of Bromotubercidin Treatment (5 μg/ml) on Biosynthesis in Unfused Cells

(*a*) The rate of RNA synthesis in melanocytes treated with bromotubercidin for various times, based on three determinations at hourly intervals

Treatment	Rate of incorporation of ^{3}H-uridine (*cpm/μg protein/hr*)	Per cent control
Nil	133	
During treatment (1, 5, or 9 hr)	22	16
After treatment for		
1 hr	180	132
5 hr	110	83
9 hr	100	75

(*b*) Melanocyte RNA, DNA and protein synthesis after 6 hr of treatment and further cultivation in the absence of bromotubercidin

Tracer	Hr after starting treatment	Incorporation (cpm/μg protein)		Per cent control
		Control	Treated	
^{3}H-Uridine	6	795	54	19
	8	1000	395	39
	10	1295	604	47
	12	1521	1000	56
^{3}H-Thymidine	6	475	355	75
	8	646	490	75
	10	808	500	62
	12	1008	521	51
	14	1132	630	54
^{3}H-Leucine	6	220	150	68
	8	295	174	59
	10	362	205	57
	12	440	238	55

(*c*) Macrophage RNA and protein synthesis after 4 hr of treatment and further cultivation in the absence of bromotubercidin

Tracer	Hr after Starting treatment	Incorporation (cpm/μg protein)		Per cent control
		Control	Treated	
^{3}H-Uridine	4	731	86	12
	5	868	334	39
	6	1051	500	48
^{3}H-Leucine	4	25	117	68
	5	32	21	66
	6	37	28	75

thesis reversibly in melanocytes and macrophages for several hours, without extensive depression of protein or DNA synthesis.

(*2*) *Bromotubercidin treatment before cell fusion:* Melanocytes or macrophages were treated with 5 μg/ml bromotubercidin for 1, 3, 5, or 7 hr, washed well, and fused 1 hr later. Macrophage DNA synthesis in heterokaryons after a 7 hr pretreatment of either melanocytes or macrophages is shown in Fig. 7. After melanocyte pretreatment the initiation of macrophage DNA synthesis was delayed until 10 hr after fusion. 40–65% of the melanocyte nuclei were labeled throughout this experiment, though often less heavily than in untreated controls.

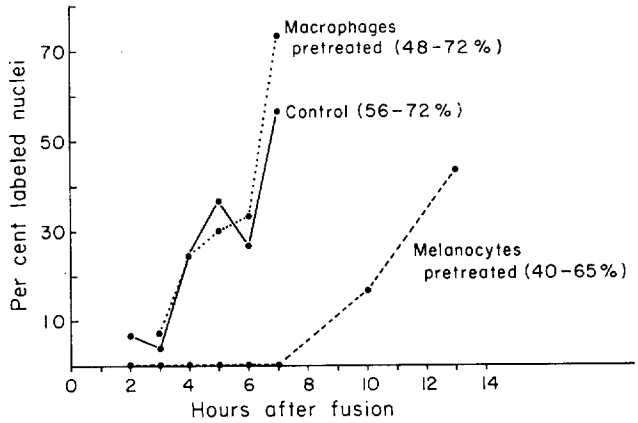

FIG. 7. Macrophage DNA synthesis in 1:1 heterokaryons after treating either melanocytes or macrophages with 5 μg/ml of bromotubercidin for 7 hr before fusion. Per cent of labeled melanocyte nuclei given in brackets.

RNA synthesis was evaluated by radioautography 1–2 hr after fusion. Treated melanocytes had 30–50% as many grains as untreated melanocytes, indicating that melanocyte RNA synthesis had not yet fully recovered. The macrophage nuclei of heterokaryons derived from pretreated melanocytes had 81% as many grains as those in untreated controls (13.7 ± 4.9 compared with 16.8 ± 9.0). Cross-toxicity had therefore not occurred in these cells. Shorter pretreatment of melanocytes, for 5 or 3 hr, caused a similar delay in macrophage DNA synthesis, but the effect was reversed more rapidly. After 1 hr of pretreatment no effect on macrophage DNA synthesis could be detected.

These experiments indicated that inhibition of melanocyte RNA synthesis with bromotubercidin reversibly blocked macrophage DNA synthesis without affecting macrophage RNA synthesis. Melanocyte RNA synthesis by itself was therefore essential for macrophage DNA synthesis and macrophage RNA synthesis could not substitute for the melanocyte in this respect.

After macrophage pretreatment, heterokaryon DNA synthesis was indistinguishable from the control (Fig. 7). The treated macrophage nuclei in heterokaryons had 61% as many RNA grains as those in untreated controls (11.0 ± 5.6 compared with 16.8 ± 9.0). Melanocyte nuclear grains and morphology showed that no cross-toxicity had occurred from the treated macrophage nucleus.

These findings are also compatible with the previous conclusion that macrophage RNA synthesis is not required for DNA synthesis.

DISCUSSION

Mouse peritoneal macrophages do not make DNA under the present conditions of in vitro cultivation. (10) These cells can, however, be induced to make DNA in vitro when treated with conditioned medium derived from L-cells, or when infected with polyoma virus (11, 12). In the present studies DNA synthesis was stimulated in dormant macrophage nuclei by fusion with rapidly proliferating melanoma cells. This stimulation occurred even when several macrophages were fused with a single melanocyte and often resulted in synchronous DNA synthesis in all the nuclei, a common finding in other multinucleated cells (1, 6, 13, 14). The nondividing mouse peritoneal macrophage, therefore, seems be be lacking in elements which induce DNA synthesis rather than actively inhibiting this process (15).

Macrophage DNA synthesis lags 2–3 hr behind that of the melanocyte nucleus in the same cytoplasm and the chick erythrocyte nucleus responds even more slowly, whether in melanocyte or HeLa cytoplasm (8). The delay in the onset of macrophage DNA synthesis is not an artifact due to prolonged fusion, which is often completed in ½ hr. Moreover, HeLa nuclei in G_1 have been reported to start DNA synthesis within an hour of fusion with other HeLa cells which are already in S (16).

The heterochromatin content of macrophage and erythrocyte nuclei could account for the different kinetics of initiating DNA synthesis in heterokaryons. DNA replication in heterochromatin can occur later than in euchromatin, even when present in the same nucleus (6). Bolund and his coworkers have shown that reactivation of the chick red cell nucleus in HeLa cytoplasm is associated with marked changes in its chromatin structure, revealed by changes in thermal stability and the binding of acridine orange (8). Perhaps condensed chromatin undergoes similar changes in structure when macrophage nuclei swell in a heterokaryon, making it competent to respond to cytoplasmic stimuli which induce DNA synthesis.

Actinomycin prevents initiation of macrophage DNA synthesis most effectively when treatment of heterokaryons is started 1–4 hr before DNA synthesis. Although the nature and function of the RNA species which are involved in the initiation of DNA synthesis are completely obscure, higher doses of actino-

mycin are needed to prevent the initiation of DNA synthesis than to inhibit the bulk of RNA synthesis. These results are compatible with a model in which new RNA species, perhaps messenger RNA, made some hours before DNA synthesis, determine a new round of DNA replication.

Heterokaryons, therefore, conform to other model systems for stimulated DNA synthesis, in which early changes in RNA synthesis take place and where inhibitors of RNA synthesis delay or abolish the entry of cells into S (6, 17). There is, however, a striking difference between other models and macrophage–melanocyte heterokaryons in that the prereplicative period is reduced from the usual 12–15 hr or longer, to 2–3 hr (17, 11). This difference is presumably due to the direct use of melanocytic products by the macrophage nucleus, bypassing many steps essential for DNA synthesis in unfused cells.

Evidence has been presented that only the melanocyte nucleus provides the RNA species necessary for macrophage DNA synthesis in heterokaryons. Bromotubercidin pretreatment of melanocytes before fusion selectively inhibited macrophage DNA synthesis after fusion, without affecting its RNA synthesis. Other evidence also argues that the stimulation in macrophage RNA synthesis is not critical for subsequent DNA synthesis. Macrophage DNA synthesis is most sensitive to actinomycin treatment before macrophage RNA synthesis is much increased and macrophages which have been treated with high doses of actinomycin before fusion make DNA, but little RNA, afterwards.

The use of inhibitors to achieve selective effects in heterokaryons presents some interesting problems. Since the cell fusion process itself is independent of DNA, RNA or protein synthesis, it is possible to treat cells with various inhibitors before, as well as at the time of, fusion (18). An important complication arises if the inhibitor diffuses from treated to untreated regions of a heterokaryon. Actinomycin, which is noncovalently bound to DNA, readily attacks untreated nuclei in heterokaryons (19). However, bromotubercidin is incorporated into RNA and does not give rise to such cross-toxicity.

Once the macrophage nucleus becomes activated in the heterokaryon it becomes less sensitive to the action of actinomycin. Macrophages which make no RNA after treatment with 1 μg/ml actinomycin showed unimpaired stimulation of RNA synthesis after fusion with untreated melanocytes. Ringertz and his coworkers contend that RNA synthesis and the binding of actinomycin D vary in different cells in parallel with the functional state of their chromatin (20). Activation of a pretreated macrophage nucleus in a heterokaryon may make new sites, unoccupied by actinomycin, available for RNA synthesis.

Studies to be reported in a subsequent communication will describe the requirements for protein synthesis and the use of synchronized melanocytes in the initiation of macrophage DNA synthesis.

SUMMARY

Mouse peritoneal macrophages, which do not synthesize DNA in vitro, were fused with melanocytes, a mouse cell strain which proliferates rapidly in vitro. DNA synthesis was induced in macrophage nuclei 2–3 hr after fusion and occurred irrespective of the number of macrophage nuclei present per melanocyte nucleus in each heterokaryon. 50–80% of macrophage nuclei initiated DNA synthesis in the 3–7 hr period after fusion. The activation of most 11–12-day chick red cell nuclei in melanocyte cytoplasm took longer than 10 hr. The lag before DNA synthesis may reflect the heterochromatin content of each nucleus.

Studies with actinomycin showed that heterokaryon RNA synthesis was essential for subsequent macrophage DNA synthesis. This RNA was synthesized 1–4 hr before the DNA and was unlikely to be ribosomal RNA, since it was insensitive to <0.1 μg/ml actinomycin.

Melanocytes and macrophages were treated before fusion with actinomycin and bromotubercidin to bring about a more selective inhibition of RNA synthesis. Macrophages pretreated for 1 hr with 5 μg/ml of actinomycin showed less than 20% of control RNA synthesis in the first 4 hr after fusion, but a normal activation of macrophage DNA synthesis. Pretreatment of melanocytes for 3–7 hr with 5 μg/ml bromotubercidin, a reversible inhibitor of RNA synthesis, prevented macrophage DNA synthesis without affecting macrophage RNA synthesis in the heterokaryons (81% of control). These studies showed that only melanocyte RNA synthesis was essential for the production of macrophage DNA.

The exposure of one cell partner to actinomycin before fusion caused cross-toxicity of the untreated nucleus after fusion. Bromotubercidin, an adenosine analogue which is incorporated into RNA, did not give rise to such cross-toxicity after fusion.

Once the macrophage nucleus becomes activated in the heterokaryon it becomes less sensitive to the action of actinomycin.

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