MACROPHAGE-MELANOMA CELL HETEROKARYONS

III. The Activation of Macrophage DNA Synthesis. Studies with Inhibitors of Protein Synthesis and with Synchronized Melanoma Cells*

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Macrophages, which do not usually divide during in vitro cultivation, made DNA after virus-induced fusion with a strain of mouse melanoma cells which does proliferate in vitro (1, 2).

The macrophage heterokaryons presented several advantages as a model system to study the initiation of DNA synthesis: unfused macrophages made no DNA and the onset of DNA synthesis was rapid, synchronous, and reproducible so that it was possible to study the requirements for DNA synthesis in the macrophage nucleus. The induction of macrophage DNA synthesis depended upon heterokaryon RNA synthesis contributed entirely by the melanoma nucleus.

In the present studies we examine the role of heterokaryon protein synthesis in activating macrophage DNA synthesis and again evaluated the contribution of each parent cell. We also studied the effect on DNA synthesis of fusing melanoma cells at different stages of their cell cycle.

Materials and Methods

Cell Culture and Virus-Induced Fusion.—The methods of cell culture and virus-induced fusion have been described previously (1). 2×10^5 mouse peritoneal macrophages were cultivated on 12-mm cover glasses for 1–2 days. Exponentially growing or synchronized melanoma cells were then added to the macrophage monolayer. 500 hemagglutinating units of ultraviolet irradiated Sendai virus were used to initiate fusion 1 hr later.

Inhibitors.—Cycloheximide and streptovitacin A were obtained from Dr. S. Silverstein of The Rockefeller University. Concentrated stock solutions were stored at −20°C and thawed before use. Actinomycin was used as before (2).

In order to study the effects of inhibitors on unfused cells, we measured the incorporation of leucine-^{3}H, uridine-^{3}H, and thymidine-^{3}H into trichloroacetic acid (TCA)-insoluble[1] product. In some experiments the cumulative incorporation of precursor was measured during drug treatment as well as subsequently; in others, only after washing out the drug.

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[1] *Abbreviations used in this paper:* PCA, perchloric acid; TCA, trichloroacetic acid.

Inhibitors were also used to treat cells either selectively, before fusion, or after fusion (2). The preparations were then washed three times with medium 199 (Microbiological Associates, Inc., Bethesda, Md.) to remove excess inhibitor. DNA, RNA, and protein synthesis were measured in fused preparations by radioautography (1).

Melanoma Cells.—Synchronized melanoma cells were obtained by shaking cells in mitosis from monolayer cultures. Melanoma cells, growing exponentially in Falcon T-75 flasks (Falcon Plastics, Los Angeles, Calif.), were rinsed twice with a solution of phosphate-buffered saline which lacked divalent cations. These washes were discarded. Flasks were next shaken vigorously and dislodged cells collected and spun down. These cells were gently resuspended in a small volume of culture medium 199 with 20% newborn calf serum and 5×10^3–1×10^4 cells added to 1-day old macrophage monolayers. Two T-75 flasks (Falcon) yielded enough cells for 20–40 cover slips. After 1–2 hr incubation to permit attachment of the melanoma cells, the preparations were gently washed and incubated further. The cocultivated cells were fused by the usual method at different times after replating the melanoma cells. Many doublets, cells in telophase, were seen soon after replating the melanoma cells. The degree of cell synchrony was estimated by radioautography after serial 2-hr pulses with thymidine-^{3}H.

Inhibitors were also used in experiments with synchronized melanoma cells, in which case the inhibitor was added at different times after replating the melanoma cells, i.e., at different stages of the melanoma cell cycle. The inhibitor was either added at the time of fusion or 2 hr afterwards and treatment was then maintained continuously until the end of the experiment.

Characterization of the Perchloric Acid (PCA)-Soluble Pool after Incubation in Thymidine-^{3}H.—Macrophages or melanoma cells were cultivated in 60-mm Falcon plastic tissue culture dishes for 1 day. The cells were washed twice with warm phosphate-buffered saline and exposed for 4 hr to 4 μCi/ml thymidine-^{3}H in medium 199 + 10% newborn calf serum. After two washes in ice-cold saline, the cells were scraped in saline and mixed with 5% ice-cold perchloric acid. The PCA-soluble fraction was washed twice with 1:3 ethanol:ether and then brought to pH 5 with 6 N KOH. The K perchlorate was spun down and the supernatant fraction concentrated *in vacuo*. The PCA-soluble constituents were separated by high voltage paper electrophoresis at pH 3.5, eluted from the paper, and their radioactivity measured in a scintillation counter.

RESULTS

The kinetics of activation of macrophage DNA synthesis have been described previously (2). A wave of DNA synthesis started in the previously dormant macrophage nuclei 2–3 hr after fusion with exponentially growing melanoma cells. 50–80% of the macrophage nuclei in heterokaryons made DNA within 6–8 hr of fusion. The role of protein synthesis in this activation process was studied with the aid of inhibitors of protein synthesis.

Protein Synthesis and the Activation of Macrophage DNA Synthesis.—The effect of cycloheximide on macromolecular synthesis in unfused cells was first determined (Table I). 91–97% of protein synthesis could be inhibited in both melanoma cells and macrophages during a 3 hr period of treatment with 1–5 μg/ml cycloheximide. This effect was reversible for both cell types. Melanoma cell DNA synthesis was depressed 54–73% by such cycloheximide treatment and RNA synthesis inhibited to a maximum value of 45%.

Cycloheximide was next used to inhibit protein synthesis in heterokaryons. Fig. 1 *a* illustrates the effect of 3 hr treatment with different doses of cycloheximide on the activation of macrophage DNA synthesis in heterokaryons. In

TABLE I

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

Tracer	Dose cycloheximide	Time after start of cycloheximide treatment	Incorporation (cpm,/μg protein)		Per cent inhibition	
			MEL‡	MAC‡	MEL	MAC
	μg/ml	*hr*				
Leucine-^{3}H	0	3	160	84		
		4	215	128		
		6	320	180		
	0.1	3	40	38	75	55
		4	110	80	49	38
		6	205	160	36	11
	1.0	3	9	78	94	91
		4	66	34	63	73
		6	155	68	52	62
	5.0	3	6	2.8	96	97
		4	50	34	52	73
		6	135	68	60	62
Uridine-^{3}H	0	3	230	310		
		5	430	420		
		7	608	600		
	0.1	3	160	387	30	−25
		5	355	400	17	5
		7	600	500	1	17
	1.0	3	137	226	40	27
		5	301	255	30	39
		7	580	390	5	35
	5.0	3	135	220	41	29
		5	250	230	42	45
		7	432	390	30	35
Thymidine-^{3}H	0	3	170			
		5	250			
		7	361			
	0.1	3	95		44	
		5	142		43	
		7	206		43	
	1.0	3	79		54	
		5	66		73	
		7	125		65	
	5.0	3	53		69	
		5	66		73	
		7	125		65	

* Cells were treated with cycloheximide for 3 hr in the presence of radioactive precursor, washed three times, and then incubated in fresh precursor without drug.

‡ MEL, melanoma cell; MAC, macrophage.

contrast with the rapid and efficient stimulation of DNA synthesis in untreated heterokaryons, cycloheximide treatment diminished and delayed this process in a dose related fashion. Cycloheximide treatment did not diminish the number of melanoma nuclei making DNA in this experiment since 60–72% of nuclei were labeled with thymidine in both the treated and control groups. Some macrophage nuclei which made no DNA as a result of cycloheximide treatment were nevertheless enlarged.

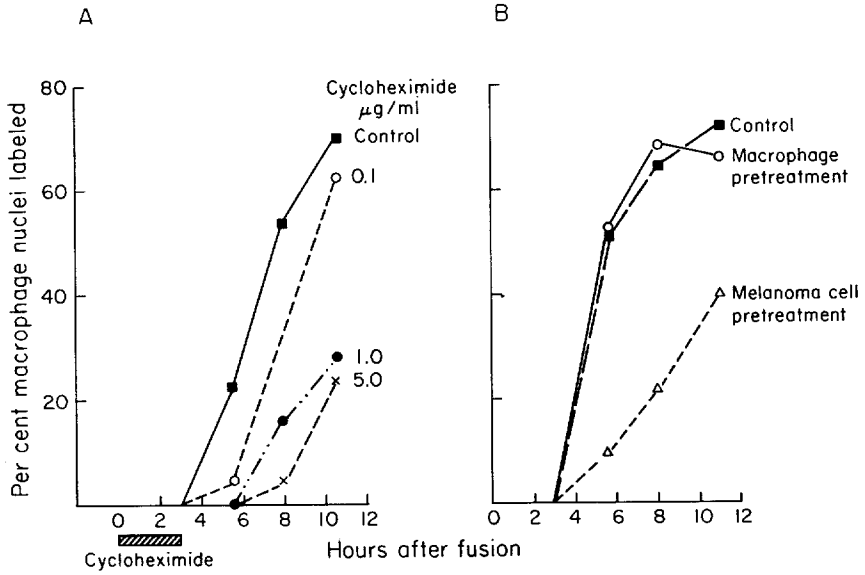

FIG. 1. The activation of macrophage DNA synthesis in heterokaryons after the inhibition of protein synthesis. (*a*) Cycloheximide treatment after fusion. (*b*) Streptovitacin treatment before fusion.

To find out which cell made the proteins required for macrophage DNA synthesis, each cell was treated, before fusion, with streptovitacin A, an irreversible inhibitor of protein synthesis. Preliminary experiments to determine the effect of streptovitacin A on unfused cells are shown in Table II. Treatment with 2 μg/ml for 1 hr suppressed 79–86% of protein synthesis in both macrophages and melanoma cells. This effect was not reversed during a subsequent 4 hr period of incubation without inhibitor. Maximal inhibition of protein synthesis was associated with 74% inhibition of DNA and 45% inhibition of RNA synthesis.

The effect of streptovitacin pretreatment before cell fusion is shown in Fig. 1 *b*. Macrophage DNA synthesis was unaffected by pretreating macrophages with 2 μg/ml streptovitacin, but melanoma cell pretreatment at 2 μg/ml clearly

TABLE II

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

Tracer	Dose streptovitacin	Time after washing out drug	Incorporation (cpm/μg protein)		Per cent inhibition	
			MEL	MAC	MEL	MAC
	μg/ml	*hr*				
Leucine-^{3}H	0	2	108	180		
		4	222	300		
	0.1	2	76	84	30	53
		4	156	169	30	43
	0.5	2	44	N.D.	59	N.D.
		4	80	N.D.	64	N.D.
	1.0	2	26	26	76	86
		4	66	64	70	79
	2.0	2	18	N.D.	84	N.D.
		4	46	N.D.	79	N.D.
	5.0	2	16	17	85	90
		4	42	40	81	87
	10.0	2	12	N.D.	89	N.D.
		4	38	N.D.	83	N.D.
Uridine-^{3}H	0	2	220	194		
		4	444	400		
	0.1	2	212	294	4	−50
		4	380	478	14	−20
	0.5	2	182	N.D.	17	N.D.
		4	300	N.D.	32	N.D.
	2.0, 5.0, 10.0	2	124	202	44	−1
		4	248	248	45	38
Thymidine-^{3}H	0	4	170			
		7	320			
	0.1	4	175		8	
		7	290		9	
	1.0	4	70		63	
		7	110		66	
	2.0, 10.0	4	50		74	
		7	100		69	

* Cells were treated with streptovitacin for 1 hr, washed 3 times, and incubated in radioactive precursor without inhibitor.

‡ N.D., not done.

TABLE III

*Leucine Labeling after Streptovitacin Pretreatment**

Pretreatment	1:1 Heterokaryons					Unfused macrophages			
	Melanoma nucleus		Macrophage nucleus		Cytoplasm	Nucleus		Cytoplasm	
	Mean	SE	Mean	SE		Mean	SE	Mean	SE
Control	84.4	9.9	31.5	3.3	>200	4.5	0.76	15.3	2.0
Macrophages	96.9	3.3	34.6	2.2	>200	1.3	0.56	5.0	1.0
Melanoma cells	33.0	3.8	12.3	1.9	75.1	5.5	0.87	19.6	2.2

* Melanoma cells or macrophages were treated with 2 μg/ml streptovitacin A for 1 hr before fusion. Preparations were exposed to L-leucine-^{3}H, 10 μCi/ml, 1–2 hr after fusion. Grain counts for 20 cells.

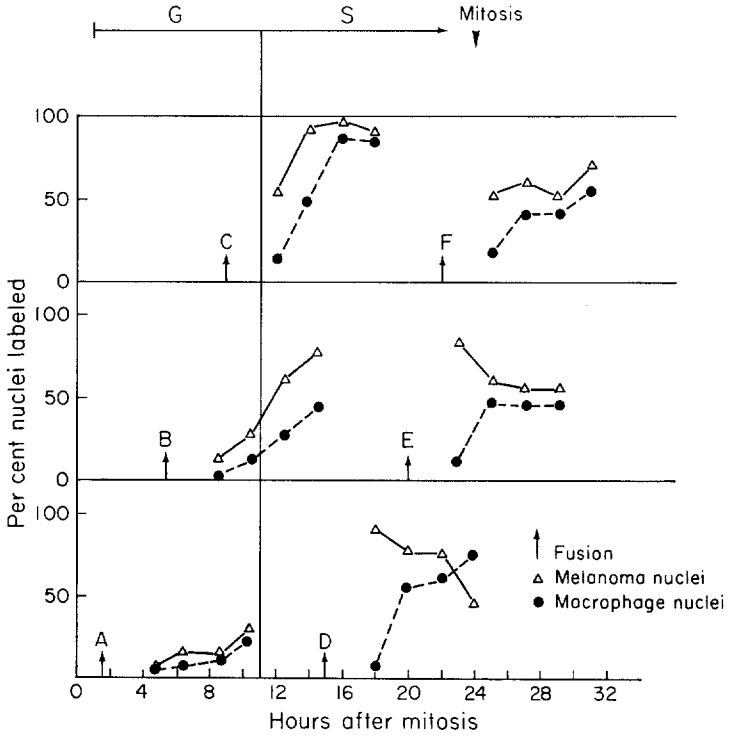

FIG. 2. DNA synthesis in heterokaryons after fusion of macrophages with synchronized melanoma cells.

depressed the activation of macrophage DNA synthesis. A dose related effect on macrophage DNA synthesis could be shown when melanoma cells were pretreated with 0.5–2 μg/ml, whereas even 20 μg/ml pretreatment of macrophages had no effect on subsequent activation. 52–72% of melanoma cell nuclei were labeled with thymidine in all these experiments.

Cells were also incubated with leucine-^{3}H after streptovitacin pretreatment to measure protein synthesis in fused preparations (Table III). In the untreated control the macrophage nuclei inside heterokaryons were more heavily labeled than those in unfused cells (31.5 vs. 4.5 grains/nucleus). After streptovitacin pretreatment of macrophages, the heterokaryon grain counts were undiminished in all cell compartments whereas cytoplasm and both nuclei showed reduced labeling after melanoma cell pretreatment. Macrophage DNA synthesis could therefore be depressed by selective inhibition of melanoma protein synthesis.

TABLE IV

DNA Synthesis in Macrophage-Melanoma Cell Heterokaryons after Inhibiting RNA and Protein Synthesis at Different Times during the Melanoma Cell Cycle

Inhibitor	Time of fusion*	Start of inhibitor treatment‡	Maximum % thymidine labeling in heterokaryon§	
			Macrophage nuclei	Melanoma nuclei
	hr	*hr*		
	9 (or earlier)	—	86	96
	15	—	60	70
Actinomycin (2 μg/ml)	2	4	0	6
	5	7	8	20
	7	9	40	70
	11	13	70	70
	15	15	20	73
Cycloheximide (5 μg/ml)	4	6	2	8
	7	9	44	84
	10	12	80	90
	15	15	37	87
Streptovitacin A (2 μg/ml)	15	15	33	73

* Mitotic melanoma cells replated on macrophage monolayers at time zero.

‡ Inhibitors were present continuously until the end of the experiment.

§ 2-hr thymidine pulses until 21 hr after replating melanoma cells.

Fusion Studies with Synchronized Melanoma Cells—When melanoma cells in mitosis were replated on macrophage monolayers, they entered S in a synchronous fashion 11 hr later and 98% of melanoma cells labeled with thymidine 4–6 hr thereafter (Fig. 2). The exit from S into G_2 was less synchronous and the peak of mitosis occurred 23 hr after replating. The cell cycle after replating was thus considerably longer than the 12 hr doubling time observed during exponential growth of melanoma cells.

Fig. 2 also shows DNA synthesis in heterokaryons after fusing macrophages with synchronized melanoma cells at different times during the melanoma cell

cycle. The onset of melanoma cell DNA synthesis was not affected by exposure to Sendai virus or by fusion with a macrophage. When the melanoma cells were in G_1 at the time of fusion (Fig. 2 *a* and *b*), macrophage DNA synthesis was delayed until after the onset of melanoma cell S. When fusion occurred late in melanoma G_1 (Fig. 2 *c*) or in mid-S (Fig. 2 *d*), macrophage DNA synthesis showed the basic 3 hr lag in initiation and then proceeded rapidly to

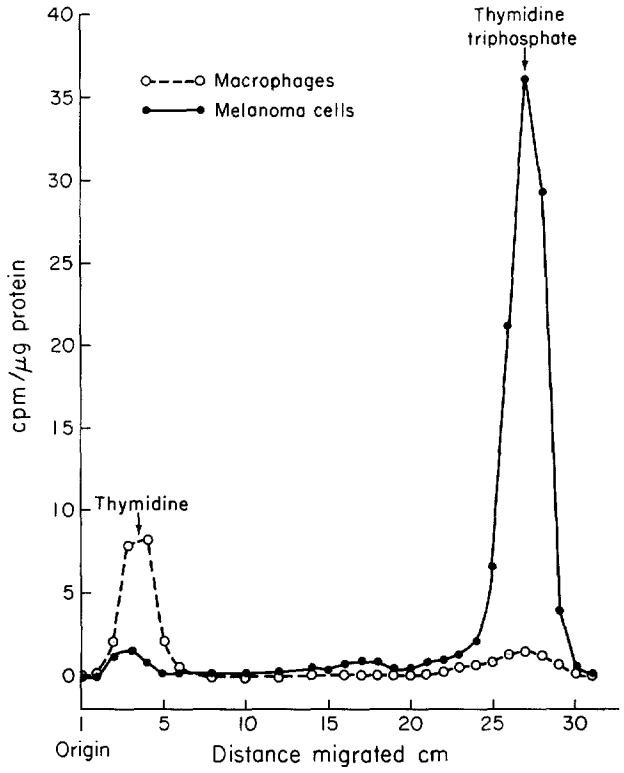

FIG. 3. The PCA-soluble products of thymidine-^{3}H in unfused cells.

involve 80–85% of the macrophage nuclei. As the melanoma cells reached the end of S (Fig. 2 *e*), fewer macrophage nuclei entered S after fusion, until the start of the next melanoma cell cycle (Fig. 2 *f*).

These experiments showed clearly that macrophage DNA synthesis was under the control of the melanoma cell cycle. The macrophage nucleus, however, still lagged in its response even after fusion with melanoma cells in mid-S.

Protein and RNA synthesis were next inhibited at different times during the melanoma cell cycle (Table IV). DNA synthesis was prevented in both the

melanoma and macrophage nuclei if actinomycin or cycloheximide treatment was started in mid-G_1 (4–7 hr). When inhibitor treatment was delayed till late G_1 (9 hr), more melanoma than macrophage nuclei initiated DNA synthesis. Both nuclei achieved maximum initiation of DNA synthesis if macrophages were fused with melanoma cells in S and inhibition delayed for 2 hr. However, when the heterokaryons were immediately treated with inhibitors of RNA or protein synthesis (fusion at 15 hr), the number of labeled macrophage nuclei was reduced selectively.

The PCA-Soluble Products of Thymidine-^3H in Unfused Cells.—It was apparent that macrophage DNA synthesis depended, in the main, on precursor products made by the melanoma cell. Could unfused macrophages by themselves convert thymidine-^3H to thymidine triphosphate for incorporation into DNA? Macrophages and melanoma cells showed quite different profiles of their perchloric acid-soluble thymidine products (Fig. 3). Thymidine taken up by the macrophage remained unphosphorylated, whereas it was recovered mainly as thymidine triphosphate from the melanoma cells. Macrophages and melanoma cells therefore differ strikingly in thymidine kinase activity.

DISCUSSION

The experiments with cycloheximide showed that protein synthesis was required for the macrophage nucleus to enter S; the studies with streptovitacin A suggested that these proteins came from the melanoma cell. These two compounds are closely related in structure and mode of action, although the detailed mechanism of inhibition has not been determined (3, 4). In addition streptovitacin A becomes irreversibly bound within cells.

Although macrophage and melanoma cell cytoplasm mix intimately after fusion, macrophage pretreatment with streptovitacin did not inhibit heterokaryon protein or DNA synthesis. Its selective action argues against free diffusion of this drug within the fused cell. Emetine hydrochloride, a different irreversible inhibitor of protein synthesis (5), did abolish heterokaryon protein synthesis after macrophage pretreatment (unpublished observations).

The studies with synchronized melanoma cells showed, in agreement with findings in other heterokaryon systems (6), that the S phase predominates over G_0. DNA synthesis initiation in both the melanoma and macrophage nuclei requires RNA and protein synthesis. The novel aspects of the present study include the characteristic lag in macrophage response and the continued dependence on RNA and protein synthesis at melanoma mid-S to initiate macrophage DNA synthesis.

Macrophages and melanoma cells differ in thymidine kinase activity and thymidine triphosphate content after incubation in thymidine-^3H. This distinction is typical of resting and proliferating cells, but is not considered to be of primary importance in regulating DNA synthesis (7, 8).

All the evidence therefore suggests that the melanoma cell, in following its

own cell cycle, provides RNA, proteins, and precursors which act upon a relatively inert macrophage nucleus to induce DNA synthesis. It is likely that proteins, ions, and DNA precursor enzymes are transported into the macrophage nucleus during the early phase of swelling. The distribution of leucine-^{3}H label in control and streptovitacin pretreated cells is compatible with this hypothesis and also argues against drug-insensitive protein synthesis within the macrophage nucleus itself (9, 10). The transport of cytoplasmic proteins into heterokaryon nuclei would account, in part, for their increase in dry mass during activation (11). Cytoplasmic factors, including proteins, are also necessary to support DNA synthesis in isolated nuclei (12, 13) and in frog somatic cell nuclei which have been transplanted into egg cytoplasm (14, 15). Labeled proteins previously synthesized by melanoma cells can be found in the macrophage nuclei of heterokaryons prepared in the presence of protein synthesis inhibitors (unpublished radioautographic observations). Future progress in characterizing the melanoma proteins which initiate macrophage DNA synthesis will require their characterization from macrophage nuclei isolated from heterokaryons.

However, the macrophage nucleus cannot be completely passive during the activation process. In the absence of a requirement for new macrophage RNA and protein synthesis, the lag in response of the macrophage nucleus could be because of physical changes which prepare the DNA template for replication (16). The nature and duration of these changes may be related to the heterochromatin content of the macrophage nucleus (2, 8).

This macrophage heterokaryon system therefore makes it possible to separate the role of template and inducing signals in bringing about DNA synthesis in G_0 cells.

SUMMARY

Dormant macrophage nuclei initiate DNA synthesis 2–3 hr after fusion of macrophages with exponentially growing melanoma cells. Cycloheximide treatment (1–5 μg/ml) of heterokaryons during the preceding lag period inhibits the initiation of macrophage DNA synthesis, in a reversible fashion. Each type of cell was also treated with streptovitacin A, an irreversible inhibitor of protein synthesis. Pretreatment of the melanoma cells (0.5–2 μg/ml), 1 hr before fusion, inhibited the induction of macrophage DNA synthesis in heterokaryons, whereas pretreatment of macrophages (1–20 μg/ml) had no effect. Melanoma cell pretreatment reduced the incorporation of leucine-^{3}H into the cytoplasm and nuclei of heterokaryons, whereas macrophage pretreatment had no effect. These experiments suggested that melanoma proteins played an important role in the initiation of macrophage DNA synthesis.

The relationship between the melanoma cell cycle and macrophage DNA synthesis was studied with synchronous melanoma cells. If the melanoma cells

were in S phase at the time of fusion, macrophage DNA synthesis occurred 2 hr later. However, the fusion of melanoma cells in G_1 delayed macrophage DNA synthesis until the melanoma nuclei had entered S. Experiments with actinomycin and cycloheximide showed that RNA and protein, essential to achieve DNA synthesis in the macrophage nucleus, were made during late G_1 as well as S.

Melanoma cells and macrophages differ in their radiolabeled acid-soluble products after incubation in thymidine-^{3}H. Thymidine taken up by the macrophage remained unphosphorylated, whereas it was recovered mainly as thymidine triphosphate from melanoma cells.

These findings, as well as those reported previously, suggest that the melanoma cell provides the RNA, protein, and precursors which initiate macrophage DNA synthesis. In the absence of a requirement for new macrophage RNA and protein synthesis, other changes must be responsible for the 2 hr delay in DNA synthesis. These may involve physical changes in DNA, associated with swelling, as well as the transport of melanoma products into the macrophage nucleus.

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