geminal ganglia showed a distinct, often granular specific fluorescence of varying intensity (Fig. 1A). Almost all positive cell bodies seemed to belong to the small-sized population of ganglion cells. Fiberlike structures with a granular fluorescence were also observed within the ganglia (Fig. 1A). In the sciatic nerve, bundles of thin fluorescent, probably unmyelinated, fibers could be observed. In the skin, thin fluorescent fibers could be seen in the connective tissue, often just beneath the epithelium. In the nasal mucosa, a dense plexus of substance P–positive fibers was observed (Fig. 1B). In the spinal cord, an extremely dense network of positive fibers was seen in the substantia gelatinosa and, although less dense, also around the central canal and in the ventral horns. Brain areas rich in fluorescent fiber systems were, among others, the substantia gelatinosa trigemini, part of the nucleus tractus spinalis nervi trigemini, the periaqueductal central grey, part of the interpeduncular nucleus, the substantia nigra, the medial amygdaloid nucleus, and the medial preoptic area.

We have also studied the nature of the substance P–positive terminal by immunohistochemistry at the ultrastructural level and have found that the positive immunoreaction in fiberlike structures is confined to nerve endings (*15*). So far substance P–positive cell bodies have only been found in one area in the rat central nervous system, namely, in the medial habenula.

In the cat the distribution of fluorescent fibers in the spinal cord is similar to that of the rat; that is, there are distinct networks of substance P–positive fibers in the ventral horns and an extremely strong immunoreaction in the substantia gelatinosa (Fig. 1C). In the pad of the hind paw, single fluorescent-positive fibers were seen under the epithelium, and a network of positive fibers surrounded the sweat glands. Positive structures with a distribution similar to those described above could not be observed after incubation with control serum.

Our results indicate that substance P or a peptide similar to it is present in certain neurons of the spinal ganglia. Cell bodies, as well as peripheral and central nerves with a distribution corresponding to that of the peripheral and central branches of primary sensory neurons, were stained. These findings give morphological support for the view that substance P may be the transmitter substance in some of the sensory neurons of the rat and cat as postulated as a result of biochemical and neurophysiological studies (*6–8*) and of more recent experimental biochemical (*16*) and immunohistochemical (*17*) studies.

At present it is not possible to relate the substance P–containing neurons to a specific sensory modality. However, our results indicate that substance P–containing fibers are unmyelinated and that in the skin these fibers appear to constitute free nerve endings.

In all probability substance P is not confined to primary sensory neurons. In the periphery not only "free" nerve endings in the skin but also fibers surrounding the sweat glands were substance P–positive, an indication that some substance P–positive fibers have a sensory function, whereas others may have a motor function—as suggested also by the effects on gut motility (*1, 4*). In the central nervous system substance P or a peptide similar to it has been observed in cell bodies in the medial habenula and in probable nerve terminals in many brain regions not directly considered as terminal areas for sensory afferents. These results confirm earlier findings of a widespread distribution of substance P in the brain (*4–6, 14*) and indicate that it may act as a transmitter in different types of neuron systems. An excitatory effect in some brain areas has in fact been observed in electrophysiological studies (*18*). The possibility must also be kept in mind, however, that different types of substance P may be present and that the various systems demonstrating it in our study may in fact contain such cross-reacting peptides.

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Induction of F_1 Hybrid Antiparent Cytotoxic Effector Cells: An in vitro Model for Hemopoietic Histoincompatibility

Abstract. *An in vitro system has been developed in which F_1 spleen cells can generate cytotoxic activity directed specifically against parental cells. The antigenic differences detected are controlled by the* H-2D-Hh-1 *region of the murine major histocompatibility complex. The parent-F_1 combinations demonstrating F_1 antiparent activity in vitro are the same as those demonstrating F_1 rejection of parental hemopoietic grafts in vivo. Hence, the F_1 antiparent cytotoxicity test serves as an in vitro model for the recognition and effector phases of hybrid resistance to parental hemopoietic grafts and may be of value in clinical transplantation.*

Primary cytotoxic immune reactions have been generated in vitro to alloantigens (*1*), syngeneic tumor antigens (*2*), and modified autologous cell surface components (*3*). According to immunogenetic theories, such immune responses cannot be elicited by F_1 hybrid lymphocytes against parental targets because of codominant inheritance of the relevant cell surface antigens. However, grafts of hemopoietic cells fail or grow deficiently under certain circumstances in heavily irradiated mice (*4*), including F_1 hybrid recipients of parental cells (*5*). This resistance is mediated by an unusual kind of host antigraft reaction which is immunogenetically specific but insensitive to total body irradiation (*4, 5*), independent of the thymus (*4, 6*), suppressible by antimacrophage agents (*7*), and genetically determined by noncodominant *Hh* (for hemopoietic histocompatibility) genes mapping primarily in the D region

(*4, 5*) of the major histocompatibility complex (MHC). Genetic resistance to hemopoietic cells, which may also occur in dogs (*8*) and possibly in man (*9*), might be responsible for complicating clinical transplantation. A simple in vitro bioassay with predictive value would be helpful for typing and matching prospective donors and recipients and for investigating mechanisms.

The generation of primary F_1 antiparent cytotoxic reactions in spleen cell cultures, described in this report, is viewed as an important step in this direction since F_1 cells are not reactive to conventional parental alloantigens. In vitro cytotoxicity correlated consistently with resistance in vivo, even though the competence for and strength of resistance varied considerably in response to genetic, physiologic, and immunologic variables and procedures.

The predominant role of *Hh-1* (which is tightly linked to the *H-2D* region of the MHC) in specifying strong *Hh* alloantigens has been firmly established (*4, 5*). Donor cells homozygous for a given *Hh-1* allele are recognized as non-self and rejected by *H-2D-Hh-1* heterozygous F_1 recipients (*5*) or by allogeneic hosts homozygous for a different *Hh-1* allele (*4*). For example, C57BL/6 (B6) parental bone marrow is rejected by (C3H × B6) F_1 recipients. Likewise, C57BL/10 (B10) marrow grafts are rejected by B10.BR allogeneic hosts. In this report, parent-F_1 hybrid strain combinations were studied in an attempt to correlate in vivo resistance with in vitro cytotoxicity. The F_1 hybrid crosses were set up so that the offspring were heterozygotes at the MHC and other genetic loci (inbred unrelated parental strains), at the entire MHC but not at other loci (congenic-resistant parental strains), or exclusively at subregions of the MHC (congenic parental strain with recombinant MHC alleles). The F_1 mice of each type were divided into two groups and used either for in vivo grafting of parental bone marrow cells or for in vitro induction of cytotoxicity.

Graft recipients were exposed to 900 to 1000 rads of total body gamma irradiation at age 12 to 15 weeks and injected intravenously within 4 hours with 10^6 nucleated bone marrow cells from parental strain mice. Five days later, the proliferation of donor-derived cells in recipient spleens was estimated by measuring the uptake of the DNA precursor 5-iodo-2′-deoxyuridine (IUdR) labeled with radioactive ^{125}I (*4, 5, 7*). As a positive control, donor cells were transplanted into syngeneic recipients to measure proliferation under optimal conditions. As a negative control, irradiated F_1 mice not injected with marrow cells were also included in each experiment to measure baseline IUdR uptake values. Results obtained in six different F_1 hybrids, expressed as percentage of splenic uptake of IUdR promoted by grafted donor cells, are summarized in Table 1. The B6 × C3H, B6 × DBA/2, B10 × B10.A, and B10.A × B10.A(2R) hybrids were resistant to parental marrow cells of B6, B10, and B10.A(2R) donors, all of which share the *H-2D-Hh-1* determinants of the *H-2*^{b} chromosome. The first three hybrids were heterozygotes for the entire MHC, whereas the B10.A × B10.A(2R) mice were heterozygotes for *H-2D-Hh-1* and homozygotes for the other known MHC subregions. A fourth hybrid, B10.A(2R) × B10, homozygous at *H-2D-Hh-1*, was susceptible to grafts from both parents. In contrast to these results, parental marrow cells of DBA/2, B10.A, and B10.D2 mice grew well in all the F_1 recipients listed in Table 1. B10 × B10.A infant mice were phenotypically susceptible to B10 marrow grafts at 20 days of age but became resistant, presumably by maturation, by 24 days of age. B6 × C3H adult mice first treated with multiple injections of B6 spleen cells lost their resistance to B6 marrow grafts, but not to allogeneic grafts of an unrelated donor such as B10.A. The loss of resistance to B6 was not obtained with spleen cells of the other parent. In summary, these in vivo experiments performed with the same groups of mice to be used for in vitro studies verify (i) the polymorphism of *Hh-1* alleles in causing resistance to cells of one parental strain and susceptibility to those of the other, (ii) the late maturation of host reactivity against *Hh*, and (iii) the specific abrogation of resistance (*5*).

Responding F_1 hybrid spleen cells used for the in vitro studies were cultured for 5 days with irradiated parental or allogeneic stimulating spleen cells (2 : 1), according to a modified procedure already described (*3, 10*). The presence of cytotoxic cells in the 5-day cultures was assessed by incubating these cells with ^{51}Cr-labeled target cells for 4 hours (*3, 11*). The latter were transplantable ascites tumors sharing the *H-2* type of the stimulating cells (*12*). Results were expressed as percentage of specific lysis, that is, the amount of ^{51}Cr released from target cells by specially sensitized effectors (*3*).

Table 1. Proliferation of parental and allogeneic bone marrow cells grafted into irradiated F_1 hybrids from crosses between mice of unrelated or congenic-resistant strains. Nucleated donor cells (10^6) were injected into a lateral tail vein of recipients a few hours after irradiation. Splenic uptake of IUdR was determined 5 days after transplantation on 8 to 12 mice of both sexes per group. The ages of F_1 recipients at time of irradiation and grafting are listed. The classification of recipients is given by subscripts; susceptible recipients (S) are mice that are genetically unable to reject a marrow graft, whereas unresponsive mice (U) are hosts that are genetically capable of rejecting a graft but have been rendered unresponsive by prior treatment with B6 spleen cells; R, resistant; I, immature. The last column lists relevant genes that are homozygous in donor cells but heterozygous in F_1 hybrid recipients. Strains B6, B10, and B10.A(2R) share the *H-2D-Hh-1* determinants of the *H-2*^{b} chromosome; S.E., standard error.

F_1 hybrid recipients	Age	Donor cells	Splenic uptake of IUdR (mean % ± S.E.) and classification of recipients	Parent-F_1 incompatibility
B6 × C3H	Adult	B6	$0.04 \pm 0.005_R$	All of *H-2* complex and other *H* loci
		C3H	$0.70 \pm 0.06_S$	
B6 × C3H*	Adult	B6	$0.08 \pm 0.02_R$	
		C3H	$0.56 \pm 0.05_S$	
		B10.A	$0.02 \pm 0.001_R$	
B6 × C3H†	Adult	B6	$0.86 \pm 0.15_U$	
		C3H	$0.63 \pm 0.09_S$	
		B10.A	$0.05 \pm 0.007_R$	
B6 × DBA/2	Adult	B6	$0.07 \pm 0.005_R$	All of *H-2* complex and other *H* loci
		DBA/2	$0.95 \pm 0.08_S$	
B10 × B10.A	Adult	B10	$0.06 \pm 0.01_R$	All of *H-2* complex
		B10.A	$0.79 \pm 0.03_S$	
B10 × B10.A	20 days	B10	$0.58 \pm 0.04_I$	
B10 × B10.A	24 days	B10	$0.05 \pm 0.009_R$	
B10.A × B10.A(2R)	Adult	B10.A(2R)	$0.01 \pm 0.006_R$	*H-2D-Hh-1*
		B10.A	$0.62 \pm 0.03_S$	
B10.A(2R) × B10	Adult	B10.A(2R)	$0.62 \pm 0.02_S$	*H-2K, I-A, I-B, I-C*
		B10	$0.78 \pm 0.05_S$	
B10.D2 × B10.A	Adult	B10.D2	$0.59 \pm 0.06_S$	*H-2K, I-A, I-B*
		B10.A	$0.81 \pm 0.07_S$	

*Three intraperitoneal injections of 10^7 C3H spleen cells at weekly intervals; irradiation and grafting 1 to 3 weeks after the final injection. †Three intraperitoneal injections of 2×10^7 B6 spleen cells at weekly intervals; irradiation and grafting 1 to 3 weeks after the final injection.

The results obtained with six F_1 hybrid cell types of different genetic constitution are summarized in Table 2. Effector cells cytotoxic for the EL-4 target were generated in cultures of B6 × DBA/2, B10 × B10.A, B10.A × B10.A(2R), and B6 × C3H spleen cells sensitized with parental B6, B10, or B10.A(2R) cells sharing with EL-4 the determinants of the *H-2D-Hh-1* region. The EL-4 tumor targets were consistently more susceptible to lysis, although spleen cells were successfully used as *Hh* targets.

The sensitizing and target cells were homozygous in each experiment, whereas the responding cells of the hybrids were heterozygous throughout the MHC and other loci (B6 × DBA/2 and B6 × C3H), at the MHC (B10 × B10.A), or at *H-2D-Hh-1* only [B10.A × B10.A(2R)]. Effector cells for the EL-4 target were not generated in cultures of B10.A(2R) × B10 spleen cells sensitized with either parental cell type. In this experiment the responding and sensitizing cells were both homozygous at the *H-2D-Hh-1* region, although the responding cells were heterozygous at the *H-2K*, *I*, and *S* regions. By comparing the in vivo and in vitro results, it can be seen that F_1 antiparent cytotoxicity was induced in the same strain combinations which were classified as resistant in Table 1.

Effector cells cytotoxic for the P-815, L1210, and LAF-17 targets were not generated in B6 × DBA/2, B10 × B10.A, B10.A × B10.A(2R), and B10.D2 × B10.A cultures sensitized with parental DBA/2, B10.A, and B10.D2 cells. The latter shared with target cells all or part of the MHC. According to the data of Table 1, these are the strain combinations characterized by full growth of parental marrow grafts and, thus, classified as susceptible.

The F_1 antiparent cytotoxicity was specific in two ways. (i) Effector cells cytotoxic for the EL-4 target did not lyse targets of different *H-2D-Hh-1* type (Table 2). (ii) F_1 responding cells failed to generate cytotoxic effectors for the EL-4 target when stimulated with parental cells of $H\text{-}2^d$ and $H\text{-}2^a$ type. However, specific B10 × B10.A allogeneic cytotoxicity against B10.D2 and B10.D2 × B10.A allogeneic cytotoxicity against B10 were induced irrespective of the F_1 antiparent reactivity.

Infant B10 × B10.A mice were used as donors of responding cells at the ages indicated in Table 2 to strengthen the correlation between in vivo and in vitro F_1 antiparent reactivity. Cells cytotoxic for the EL-4 target were not induced in cell cultures of 20-day-old mice stimulated by parental B10 cells, despite the strong cytotoxic reaction developed by these cultures against antigens of allogeneic B10.D2 cells. In contrast, responding cells of 24- and 58-day-old F_1 mice were competent for cytotoxic reactions to both parental and allogeneic stimulating cells. Thus, the maturation of F_1 antiparent cytotoxic po-

Table 2. In vitro induction of F_1 hybrid antiparent cytotoxicity specific for gene products of the *H-2D-Hh-1* region. Spontaneous lysis of target cells in the presence of unsensitized cultured lymphocytes was 10 to 30 percent. The ratio of effector to target cells was 40 : 1. The subscripts R (resistant), S (susceptible), U (unresponsive), and I (immature) indicate the expected marrow graft susceptibility of the responding cell donors had they been used as irradiated recipients of parental marrow from the same donors as the stimulating cells (assayed on *H-2*–matched target cells). The in vivo marrow graft recipients and the in vitro responding cell donors were, in fact, taken from the same pool of animals. The second column lists ages of F_1 spleen donors at time of in vitro sensitization; the last column lists relevant genes that are homozygous in target and stimulating cells but heterozygous in responding cells; ND, not done.

Responding F_1 cells	Age	Stimulating parental cells	Specific lysis (% ± S.E.) assayed with tumor targets			Parent-F_1 incompatibility
			EL-4 ($H\text{-}2^b$)	LAF-17 ($H\text{-}2^a$)	P-815 or L1210 ($H\text{-}2^d$)	
B6 × DBA/2	Adult	B6	$29.0 \pm 2.3_R$	ND	-0.3 ± 1.3	All of *H-2* complex
		DBA/2	3.7 ± 1.9	ND	$-0.7 \pm 1.2_S$	and other *H* loci
B10 × B10.A	Adult	B10	$62.9 \pm 1.6_R$	0.4 ± 1.3	4.0 ± 2.5	All of *H-2* complex
		B10.A	-0.4 ± 0.5	-2.4 ± 1.6s	$3.8 \pm 2.6_S$	
		B10.D2	9.1 ± 0.9	ND	62.0 ± 3.4	
B10.A × B10.A(2R)	Adult	B10.A(2R)	$19.2 \pm 0.8_R$	ND	1.0 ± 2.0	*H-2D-Hh-1*
		B10.A	6.7 ± 0.8	ND	$0.0 \pm 1.9_S$	
B10.A(2R) × B10	Adult	B10.A(2R)	$0.6 \pm 2.0_S$	ND	2.6 ± 2.4	*H-2K, I-A, I-B, I-C, S*
		B10	$2.0 \pm 1.1_S$	ND	5.3 ± 2.8	
B10.D2 × B10.A	Adult	B10.D2	9.5 ± 0.9	$3.5 \pm 1.8_S$	$5.0 \pm 1.2_S$	
		B10.A	8.9 ± 1.1	$-2.9 \pm 1.9_S$	$0.6 \pm 1.3_S$	*H-2K, I-A, I-B*
		B10	77.2 ± 4.5	3.6 ± 2.0	-0.4 ± 1.6	
B10 × B10.A	20 days	B10	$6.7 \pm 0.6_I$	ND	ND	All of *H-2* complex
		B10.D2	ND	ND	47.0 ± 1.9	
B10 × B10.A	24 days	B10	$43.5 \pm 3.6_R$	ND	ND	
		B10.D2	ND	ND	35.6 ± 2.3	
B10 × B10.A	58 days	B10	$49.1 \pm 5.6_R$	ND	ND	
		B10.D2	ND	ND	60.5 ± 3.5	
B6 × C3H	Adult	B6	$34.6 \pm 3.2_R$	ND	8.8 ± 1.7	All of *H-2* complex
		C3H	4.1 ± 1.6	ND	4.1 ± 1.8	and other *H* loci
		DBA/2	8.2 ± 1.5	ND	36.0 ± 4.3	
B6 × C3H*	Adult	B6	$25.7 \pm 2.0_R$	ND	2.8 ± 0.9	
		C3H	6.8 ± 1.8	ND	-12.2 ± 0.2	
		DBA/2	-1.4 ± 2.6	ND	76.1 ± 2.8	
B6 × C3H†	Adult	B6	$3.7 \pm 1.0_U$	ND	1.1 ± 0.7	
		C3H	3.5 ± 0.8	ND	5.8 ± 1.1	
		DBA/2	9.6 ± 0.9	ND	74.6 ± 2.7	

*Three intraperitoneal injections of 10^7 C3H spleen cells at weekly intervals: spleen cells used for in vitro sensitization 1 to 3 weeks after the final injection. †Three intraperitoneal injections of 2×10^7 B6 spleen cells at weekly intervals; spleen cells used for in vitro sensitization 1 to 3 weeks after the final injection.

tential occurred at about the time of the development of competence for bone marrow graft rejection in genetically resistant infant mice (Table 1) (*4, 5*).

Responding cells of B6 × C3H mice repeatedly injected with parental C3H spleen cells retained the ability to generate both antiparental B6 and antiallogeneic DBA/2 cytotoxic effector cells (Table 2). The F_1 cells harvested from mice first treated with parental B6 cells lost their cytotoxic reactivity against B6 stimulating cells while retaining reactivity against allogeneic DBA/2 cells. Hence, the specific abrogation of hybrid resistance resulting in phenotypic susceptibility to B6 marrow grafts (Table 1) also resulted in unresponsiveness in vitro.

These experiments demonstrate that cell-mediated cytotoxicity is inducible in strain combinations for which in vitro reactions to classical *H-2K* and *H-2D* alloantigens and in vivo skin graft rejections are precluded. Such combinations are characterized, however, by strong reactivity in vivo against *Hh-1* incompatible hemopoietic grafts. No exception was noticed so far to this positive correlation, although genetic polymorphism, immunologic immaturity, and induced specific unresponsiveness were introduced in the experimental design as sources of variation to possibly dissociate resistance to bone marrow grafts and competence for in vitro cytotoxicity.

Since a population of radioresistant macrophages has been implicated as the effector of bone marrow graft rejection in vivo, presumably in cooperation with thymus-independent lymphocytes (*6, 7*), the in vitro bioassay should be instrumental in establishing to what extent the mechanisms of cytotoxicity against *Hh* differ from classical T cell–mediated lymphol-ysis. However, it is still an open question whether reactions against *Hh* are mediated by the same cell types in vitro and in vivo. Irrespective of the mechanistic aspects of this problem, this straightforward bioassay makes it possible to investigate hemopoietic histoincompatibility in species other than mice, and the role of the *Hh* system in leukemogenesis (*13*). If successful with human cells and in allogeneic combinations, the in vitro model will improve matching procedures for clinical bone marrow transplantation.

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12. Transplantable EL-4 (*H-2*^{b}, strain B6), L1210 (*H-2*^{d}, DBA/2), and LAF-17 (*H-2*^{a}, B10.A) leukemias, and P-815 (*H-2*^{d}, DBA/2) mastocytoma were serially transplanted into mice of the strain of origin. These tumors were passaged at weekly intervals by intraperitoneal injection of diluted ascitic fluid. Cells were labeled with ^{51}Cr as described (*11*).
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Differentiation of Erythroleukemic Cells in the Presence of Inhibitors of DNA Synthesis

Abstract. *Erythroid differentiation can be readily induced by butyric acid in cultured erythroleukemic cells in the presence of inhibitors of DNA synthesis and in the absence of cell division. This result appears to rule out more complex models for globin gene expression which require gene replication or cell division (or both).*

A variety of arguments have been put forward that suggest that DNA synthesis, accompanied by an appropriate number of mitoses, is necessary for the differentiation of erythroid cell precursors (*1*). Recent studies have explored this question with the use of cultured erythroleukemic cells which undergo erythroid differentiation in the presence of dimethyl sulfoxide (*2, 3*). The results of these studies suggest that two rounds of mitosis are required for erythroid induction in the presence of dimethyl sulfoxide (*4*), or that dimethyl sulfoxide must be present during, or immediately after, DNA synthesis (*5*). Such interpretations fit well with the data and with the teleologic argument that DNA synthesis results in the rearrangement of regulatory elements by exposing or activating the required set of genes.

Our own studies of this question, in which we used inhibitors of DNA synthesis and the dimethyl sulfoxide induced–erythroleukemic cell system, initially led to ambiguous results. Erythroid differentiation was invariably reduced by inhibitors of DNA synthesis, but it was never entirely quenched. In addition, it was clear that such reagents had pleiotropic effects on the inhibition of RNA and protein synthesis which were particularly severe in the presence of dimethyl sulfoxide. Thus, the observed inhibition of erythroid differentiation might have been a consequence

Table 1. Butyric acid–induced erythroid differentiation in log and stationary phase erythroleukemic cells. A cloned line of erythroleukemic mouse cells, T3C12 (*3*), was grown in F-12 medium supplemented with 10 percent calf serum (*7*). Cells in the log phase of growth were passed to a concentration of 1×10^4 cell/ml; stationary phase cells, to a concentration of 3.2×10^5 cell/ml, the usual growth limit of this cell line under these conditions. Butyric acid, 1 m*M*, was added on day zero, as indicated, and cells were assayed on day 5 for percent of benzidine-positive cells (*7*). A 0.1- to 0.2-ml sample of cells was added to a well in a Microtiter dish and mixed with 0.1 volume of freshly prepared benzidine reagent containing 0.15 percent H_2O_2, 4 percent CH_3COOH, and 0.015 percent benzidine. Approximately 200 cells of each duplicate sample were scored for benzidine-staining (positive) cells.

Cells	Cell count		Benzidine-positive cells (%)
	Initial	Final	
Log phase	1×10^4	1.2×10^5	32
Stationary phase	3.2×10^5	2.9×10^5	21