[CANCER RESEARCH 32, 98–106, January 1972]

Detection of Avian and Mammalian Oncogenic RNA Viruses (Oncornaviruses) by Immunofluorescence

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SUMMARY

The indirect immunofluorescence and immunofluorescence absorption tests were used to determine the presence and intracellular deposition of viral antigens in cells infected with avian, murine, hamster, and feline oncornaviruses. In all instances, viral antigens were restricted to the cytoplasm; nuclear fluorescence was not observed. Screening of murine neoplasms by immunofluorescence and immunofluorescence absorption showed (*a*) that murine leukemia virus and murine mammary tumor virus (MTV) were serologically unrelated; (*b*) that murine leukemia virus was widespread throughout the mouse population and occurred in a variety of tumors and leukemias of mice from both high- and low-leukemia-incidence strains; and (*c*) that MTV was restricted to a few types of neoplastic tissues, which occurred only in mice of high-mammary-tumor-incidence strains. With the immunofluorescence absorption technique, MTV, as well as murine leukemia virus, could be detected in the spleen of individual mice from high-incidence strains. The amount of viral antigen in the spleen of mice from crosses of high-mammary-tumor strains (or high-leukemia strains) with mice of low-incidence strains was intermediate between that of the parental strains. A serological comparison of murine oncornaviruses with those of the chicken, hamster, and cat revealed that (*a*) the avian and mammalian oncornaviruses are serologically unrelated to each other; (*b*) oncornaviruses from the chicken, mouse, hamster, and cat contain species-specific viral antigens that are serologically distinguishable (however, the leukemia and sarcoma viruses within a single species are antigenically indistinguishable); (*c*) the mammalian leukemia-sarcoma viruses share at least one common antigen; and (*d*) the mouse MTV is serologically unrelated to all other oncornaviruses.

INTRODUCTION

Immunofluorescence on fixed cells has proven to be a powerful tool for determining the intracellular localization of viral antigens. This method has been applied to cells infected with oncornaviruses but generally has yielded conflicting results. Whereas several investigators have reported cytoplasmic localization of viral antigens (3–5, 7, 11, 14, 15, 17, 22, 25, 30), virtually an equal number have described nuclear localization (6, 16, 18, 23, 26, 27).

We report here immunofluorescence studies with murine, feline, hamster, and avian oncornaviruses. Antisera known to contain antibodies against viral group-specific (*gs*) antigens were used to screen normal and malignant tissues. All viral-specific fluorescence reactions observed were cytoplasmic. Nuclear fluorescence was observed only with unabsorbed or inadequately absorbed antisera that were prepared in heterologous hosts. In addition, we describe the use of an IFA[4] technique to determine the presence of viral antigens in lymphoid tissues of individual mice; this method is of particular value because it lends itself to quantitative analysis and circumvents the problem of nonviral fluorescence that often is observed with lymphoid cells.

MATERIALS AND METHODS

Animals and Tumors

Most mouse and rat strains used in this study were maintained at the Sloan-Kettering Institute for Cancer Research. For the induction and designation of tumors, see Ref. 24. Strains with a high incidence of mammary tumors are C3H, DBA/2, RIII, A, and GR; these mice are all naturally infected with MTV. Strains with a high incidence of leukemia are AKR and C58; these mice are MuLV infected. The standard MuLV+ (EδG2) and MuLV– (EL4) mouse leukemias were from C57BL/6 mice. EδG2 was induced by neonatal infection with passage A Gross virus and is the G+ reference cell for the typing of mouse tissues by the cytotoxic test for G cell surface antigen (24). EL4 is a long-transplanted ascites tumor and is the G– reference cell for the cytotoxic test.

Other animals, tumors, and tissue culture lines were kindly provided by: Dr. Dmochowski (M. D. Anderson Hospital, Houston, Texas), Dr. Heston and Dr. Vhlahakis (NIH, Bethesda, Md.), Dr. Moore (Institute for Medical Research, Camden, N. J.), Dr. Ioachim (Lenox Hill Hospital, New York,

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[3] This work was supported in part by National Cancer Institute Grants CA 08748 and CA 08740.

Received July 29, 1971; accepted September 15, 1971.

[4] The abbreviations used are: IFA, immunofluorescence absorption; MTV, mammary tumor virus; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; FeLV, feline leukemia virus; HaSV, hamster sarcoma virus; AvLV, avian leukosis virus; GCSA, G cell surface antigen.

N. Y.), Dr. Medina and Dr. Liebelt (Baylor University, Houston, Texas), Dr. Hellström (University of Washington, Seattle, Wash.), Dr. Sjögren (Swedish Hospital, Seattle, Wash.), Dr. Krueger (University of Washington, Seattle, Wash.), Dr. Temin and Dr. Heidelberger (McArdle Laboratory, Madison, Wis.), Dr. Ulrich (Fibiger Laboratoriet, Copenhagen, Denmark), and Dr. Fleissner and Dr. Sanders (Sloan-Kettering Institute, New York, N. Y.).

Indirect Immunofluorescence Test (See Ref. 12)

Standard microscopic slides were cleaned in alcohol, rinsed in distilled water, and dried. Small, round pieces of filter paper (diameter, 4 mm) were moistened and spaced evenly on 8 spots of a cleaned slide. The slide was lightly sprayed (2 to 3 times) with Fluoroglide (Chemplast, Inc., Wayne, N. J.) and permitted to dry. The pieces of filter paper were removed, leaving distinctive wells surrounded by a hydrophobic film. On each slide prepared in this way 8 reactions could be performed; these include normal serum and 0.9% NaCl solution controls, as well as 6 experimental reactions.

Single-cell suspensions were prepared by mincing tissues with curved scissors (lymphoid tissues) or by trypsinization (solid tumors). Cells were washed in Earle's balanced salt solution and diluted to a final concentration of 1 to 2 X 10^6 cells/ml. One drop was placed on each of the 8 spots of a prepared slide. The cells were dried at 37° for 1 to 2 hr in an incubator, fixed for 10 min in acetone at room temperature and, if not used immediately, stored at −20°.

Tissue culture cells were attached to the slides by placing on each spot 1 drop of a viable cell suspension and were incubated overnight at 37°. Cells attached to slides by overnight growth showed more details in immunofluorescence than trypsinized cells of solid tumors. When trypsinized cells were attached by drying, the cytoplasm contracted and rounded about the nucleus; growing cells, on the other hand, were extended and showed better differentiation between the cytoplasm and nucleus.

Immunofluorescence tests were performed in 3 steps.

Step 1. Cells were incubated in a drop of antiserum dilution for 1 hr at 37° in a humidified incubator. The slides were washed twice with PBS, pH 7.2, and twice in distilled water (5-min intervals for each wash).

Step 2. Slides were dried with a hairdryer, and 1 drop of fluoresceinated antiglobulin (see below) was added to each spot. The cells were incubated for 1 hr at 37°, and the slides were washed twice in PBS and once in distilled water.

Step 3. Cells were counterstained by incubation in Evan's blue dye (0.06%) for 4 to 5 min, washed 2 times in distilled water, and dried with a hairdryer. A small drop of glycerine:PBS (1:1) was placed on each spot, and the slide was covered with a long (20 x 50 mm) coverslip. Slides prepared in this way could be stored at −20° for many months without diminution of fluorescence.

The slides were examined in a Zeiss fluorescence microscope with a dark field condenser, exciter filter (BG 12), barrier filters (50 and 44), and a high-pressure mercury bulb (HBO 200W) as the light source.

Antisera

MTV. Antiserum was prepared in rabbits immunized with density-gradient-purified MTV from RIII mouse milk (20).

MuLV. Antiserum was prepared in W/Fu X BN F_1 rats bearing a syngeneic tumor W/Fu(C58NT)D, which was originally induced by murine Gross leukemia virus (9).

FeLV. Antiserum was prepared in rabbits immunized with Tween-ether-treated, density-gradient-purified FeLV. FeLV was obtained from a line of FeLV-infected cat fibroblasts (10).

HaSV. Antiserum was prepared in W/Fu X BN F_1 rats immunized with Tween-ether-treated, density-gradient-purified HaSV. This virus was obtained from a hamster tissue culture line (B34) which continually produced this virus (21).

AvLV. Antiserum was prepared in rabbits immunized with Tween-ether-treated, density-gradient-purified avian myeloblastosis virus. AvLV was obtained from a chicken embryo fibroblast culture that was continually producing the virus. This antiserum was kindly provided by Dr. Fleissner (Sloan-Kettering Institute).

Fluorescein-conjugated Antiglobulins. Conjugates purchased from Hyland Laboratories, Inc. (Los Angeles, Calif.) were tested in immunoelectrophoresis against whole serum; all conjugates reacted only with γ-globulins. Goat anti-rabbit immunoglobulin was used at a 1:20 dilution in PBS and goat anti-rat immunoglobulin was used at a 1:10 dilution.

Preliminary Absorption Procedures for Viral Antisera

All antisera contained antibodies against normal cellular components as well as against virus-specific antigens. Antisera were therefore absorbed to eliminate the nonviral fluorescence. Antisera prepared by heterologous immunization (*i.e.*, rabbit anti-MTV) contained relatively high titers of nonviral antibodies and required extensive absorption, whereas antisera prepared by syngeneic immunization (*i.e.*, rat anti-rat MuLV+ leukemia) contained low titers and needed only minimal absorption. In some cases, diluting the antiserum past the end point of the nonviral antibodies eliminated these reactions and still permitted bright fluorescence with virus-specific antigens.

The following 2 absorption procedures were used.

***In Vitro* Absorption.** Antisera prepared against viruses isolated from tissue culture fluids were absorbed with lyophilized fetal calf serum (30 mg of powder per ml of antiserum) and with lyophilized extracts of normal liver tissues (30 mg of powder per ml of antiserum) overnight at 4°. The resulting precipitates were removed by centrifugation for 20 min at 1000 X *g*. In certain tests, the antisera prepared against MTV were absorbed overnight with an equal volume of packed washed liver homogenate, the liver cells being removed by low-speed centrifugation after absorption.

***In Vivo* Absorption.** Since in most instances it was observed that *in vitro* absorption failed to eliminate all nonviral fluorescence, heterologous antisera were absorbed *in vivo*. This was effected by the i.p. injection of antiserum into a normal animal. Antiserum was recovered the next morning by heart puncture. For antisera prepared against murine viruses (MuLV and MTV), 1 ml of antiserum was inoculated into a C57BL/6

mouse; for antisera prepared against FeLV, 10 ml of antiserum were inoculated into a kitten; for antisera prepared against AvLV, 1 ml of antiserum was inoculated into a chick.

Immunofluorescence Absorption Tests

Cell preparations were tested for viral antigens, both by the conventional indirect immunofluorescence test and by absorption of viral antisera with cellular extracts followed by testing of the absorbed antisera for residual specific antibody by immunofluorescence. Absorptions were performed with either lyophilized tissue powders or 20% tissue homogenates (sonically disrupted in 0.85% NaCl solution or Veronal buffer). For preparation of powders, tissues were homogenized in 20 volumes of distilled water in a VirTis 45, and the soluble fraction obtained after centrifugation for 2 hr at 45,000 × *g* was lyophilized and used for absorption.

For absorption tests, antisera were diluted 2 double dilutions below the end point of fluorescence and incubated overnight at 4° with the appropriate tissue powder or tissue homogenate (at a concentration of 60 mg of powder per ml of diluted antiserum or with an equal volume of homogenate). The next morning, the serum was recovered by centrifugation at 1000 × *g* for 30 min. Antigen titer refers to the maximum dilution of a 20% tissue homogenate that is capable of absorbing specific fluorescence from an antiserum tested 2 double dilutions below its end point.

RESULTS

Evaluation of Preliminary Absorption Procedures for Removal of Antibodies against Normal Tissue Constituents

Heterologous antisera contained high titers of antibodies against normal tissue antigens and required extensive absorption. Table 1 shows that *in vitro* absorption of rabbit anti-MTV serum with liver or milk was not sufficient to eliminate nonviral reactions and retain bright fluorescence. Absorption of this antiserum *in vivo* removed all traces of nonviral reactivity yet retained the bright fluorescence on MTV+ cells. All antisera prepared against virus isolated by density gradient centrifugation required *in vivo* absorption to remove nonviral fluorescence; *in vitro* absorption was not sufficient.

Antisera prepared by syngeneic immunization contained relatively low titers of antibodies against normal tissue components. Nonviral reactions could be removed by absorption with liver cells or by dilution of the antiserum past the end point of nonviral specificity (Table 2).

Table 1

Effectiveness of in vitro and in vivo absorption of the heterologous rabbit anti-MTV serum

Absorption procedure	Test cell for immunofluorescence	Fluorescence reaction at the following antiserum dilutions: Bright 2	4	8	16	32	64	128	256	512	Faint[b] 1024
Unabsorbed	MTV+ mammary tumor (C3H/An)	+	+	+	+	+	+	+	+	+	–
	MTV– leukemia (C57BL EL4)	+	+	+	+	+	+	+	+	–	–
Absorbed with liver cells[b]	MTV+ mammary tumor (C3H/An)	+	+	+	+	+	+	+	+	–	–
	MTV– leukemia (C57BL EL4)	+	+	+	+	+	–	–	–	–	–
Absorbed with milk[b]	MTV+ mammary tumor (C3H/An)	+	+	+	+	+	+	+	+	–	–
	MTV– leukemia (C57BL EL4)	+	+	+	+	+	–	–	–	–	–
Absorbed *in vivo* In a C57BL/6 mouse	MTV+ mammary tumor (C3H/An)	+	+	+	+	+	+	+	–	–	–
	MTV– leukemia (C57B EL4)	–	–	–	–	–	–	–	–	–	–

[a] Intensity of + reaction.
[b] From C57BL/6 mice.

Table 2

Effectiveness of in vitro and in vivo absorption of the rat anti-MuLV serum [W/Fu × Bn F_1 anti-W/Fu(C58NT)D]

Absorption procedure	Test cell for immunofluorescence	Fluorescence reaction at the following antiserum dilutions: Bright 2	4	8	16	32	64	128	256	512	Faint[b] 1024
Unabsorbed	MuLV+ leukemia (C57BL/6 EδG2)	+	+	+	+	+	+	+	+	+	+
	MuLV– leukemia (C57BL EL4)	+	+	+	+	–	–	–	–	–	–
Absorbed with liver cells[b]	MuLV+ leukemia (C67BL/6 EδG2)	+	+	+	+	+	+	+	+	+	+
	MuLV– leukemia (C57BL EL4)	–	–	–	–	–	–	–	–	–	–
Absorbed *in vivo* In a C57BL/6 mouse	MuLV+ leukemia (C57BL/6 EδG2)	+	+	+	+	+	+	+	+	–	–
	MuLB– leukemia (C57BL EL4)	–	–	–	–	–	–	–	–	–	–

[a] Intensity of + reaction.
[b] From C57BL/6 mice.

Localization of Viral Antigens

All immunofluorescence reactions were restricted to the cytoplasm or the cell membrane. This included tests on cells infected with AvLV, MuLV, MTV, HaSV, and FeLV. Nuclear fluorescence was not observed in any of these viral systems.

Distribution of MTV and MuLV Antigens in Neoplasms of Mice

Table 3 summarizes immunofluorescence tests with approximately 160 different neoplasms of mice. MuLV infection was widespread, occurring in tumors of mice from both high- and low-leukemia-incidence strains. MTV, in contrast, was restricted to tumors of mice from only MTV+-infected strains.

Mammary tumors from mice of MTV-infected strains were invariably positive for MTV antigens, and the same tumors were rarely positive for MuLV antigens. Leukemias from mice of high-leukemia-incidence strains were always MuLV+. Leukemias of mice from low-leukemia strains were frequently MuLV+ but only rarely MTV+ (and in these instances, the MTV+ leukemias occurred in mice of the MTV-infected strains DBA/2 and C3H). Sarcomas induced by murine sarcoma viruses were positive for MuLV antigens but not for MTV antigens. Sarcomas induced in mice by other oncogenic viruses (*i.e.*, Rous sarcoma virus, polyoma virus, and adenovirus 12) were negative for both MuLV and MTV.

MuLV and MTV antigens were not found in the majority of other tumors tested. A notable exception was the appearance of MTV in a Leydig cell tumor of BALB X A F_1 mice. This tumor contains large cytoplasmic inclusions of A particles, similar to those observed in murine mammary tumors and ML+ leukemias (22, 29).

Absorption Studies with MuLV and MTV-infected Tissues

No antigenic relatedness was observed between MTV and MuLV; this was demonstrated by: (*a*) the negative reaction of MTV antiserum on MuLV+ cells and, conversely, the negative reaction of MuLV antiserum on MTV+ cells, and (*b*) the failure to demonstrate cross-absorption between MuLV- and MTV-infected tissues (Table 4).

Table 3

The distribution of MTV and MuLV antigens in neoplasia of the mouse

Neoplasm	Mouse strain[a]	No. MTV+/no. tested	No. MuLV+/no. tested
Mammary tumors			
Spontaneous	RIII, DBA/2, CBA	3/3, 5/5, 3/3	0/3, 2/5, 0/3
	GR, A, DD	5/5, 18/18, 1/1	0/5, 0/18, 0/1
	C3H, C3Hf	28/28, 2/2	5/28, 1/2
	C57BLfC3H, BALB/c	2/2, 1/3	0/2, 0/3
Transplanted	BALB/c	0/2	0/2
Nodule outgrowth	BALB/c	0/7	0/7
Primary cultures	BALB/c, A, C3H	0/1, 1/1, 3/3	0/1, 0/1, 2/3
Tissue culture lines	C57BL X A F_1	1/1	0/1
	RIII, BALB/c	1/1, 0/6	1/1, 3/6
Leukemias			
Spontaneous and transplanted	AKR, C58, SJL/J	0/2, 0/1, 0/2	2/2, 1/1, 2/2
	DBA/2, C3Hf	2/4, 1/1	2/4, 1/1
	C57BL	0/2	0/2
Virus-induced	C57BL (Gross)	0/1	1/1
	BALB/c (Rauscher)	0/3	3/3
	BALB/c (Friend)	0/1	1/1
	Ha/ICR (Moloney)	0/1	1/1
Sarcomas			
Chemically induced	A	0/2	1/2
Virus-induced	A (Moloney)	0/6	6/6
	CF1, Ha/ICR (FBJ)	0/1, 0/1	1/1, 1/1
	A (Rous, polyoma)	0/1, 0/1	0/1, 0/1
	CBA (adenovirus 12)	0/1	0/1
Reticulum cell	SJL/J	0/5	5/5
Myeloma	BALB/c	0/7	2/7
Histiocytoma	BALB/c X DBA/2 F_1	0/2	0/2
Mast cell tumor	DBA/2	0/1	0/1
Hepatoma	C3H, C3H^{vy}fC57BL	0/1, 0/2	0/1, 0/2
Cervical tumor	C57BL	0/1	0/1
Leydig cell tumor	BALB/c X A F_1	1/1	0/1
Prostate tumor line	C3H	0/2	0/2

[a] C3H refers to substrains C3H/An, C3H/He, and C3H/Crgl; C3Hf refers to C3Hf/Bi and C3Hf/Crgl; C57BL refers to C57BL/6.

Table 4
Cross-absorption tests for MTV and MuLV antigens

Antigen used for absorption	Immunofluorescence tests[a] with absorbed antiserum		
	Anti-MTV	Anti-MuLV	
	MTV+ mammary tumor[b] (C3H/An)	MTV+ leukemia[b] (DBA/2 SL11)	MuLV+ leukemia[c] (C57BL/6 EδG2)
MTV+ mammary tumor (C3H/An)	–	–	+
MTV+ leukemia (DBA/2 SL11)	+	–	+
MuLV+ leukemia (C57BL/6 EδG2)	+	+	+
MuLV– leukemia (C57BL EL4)	+	+	+
MuLV+ spleen cells (AKR)	+	+	–
MTV+ spleen cells (A)	+	–	+

[a] +, cytoplasmic fluorescence.
[b] Tested with rabbit anti-MTV serum.
[c] Tested with rat anti-MuLV serum [W/Fu × BN F_1 anti-W/Fu(C58NT)D].

Table 5
Immunofluorescence tests showing restricted synthesis of MTV antigens in certain cell types

Antisera were prepared in W/Fu × BN F_1 rats against MTV proteins isolated from Tween-ether-treated MTV (from C3H milk) that had been chromatographically separated on Sephadex G-200 (see Ref. 22).

Strain	Cells tested	MTV C3H antigens by immunofluorescence test		
		s1,2	s3	s4,5
C3H	Mammary tumor	+	+	+
C57BLfC3H	Mammary tumor	+	+	+
C3H	Spleen	+	–	–
C3Hf	Mammary tumor	+	+	–
RIII	Mammary tumor	+	+	–
GR	Mammary tumor	+	+	–
A	Mammary tumor	+	+	–
DBA/2	Mammary tumor	+	+	+
DBA/2	ML+ leukemia	+	–	–
DBA/2	Spleen	+	–	–
(BALB × A) F_1	Leydig cell tumor	+	–	–

MTV-infected cells showed either complete or partial cross-absorption. Thus, MTV+ mammary tumors absorbed reactivity for all MTV+ tissues, whereas MTV+ leukemia cells, Leydig tumor cells, and spleen cells cross-absorbed for each other but did not absorb all reactivity for mammary tumors. This failure to obtain complete cross-absorption with MTV-infected cells was due to a difference in the synthesis of viral structural proteins in various cell types. Mammary tissues showed a productive viral infection, with the formation of extracellular virus (B particles), while the leukemia cells, Leydig tumor cells, and spleen cells were abortively infected (22). In this abortive infection, there was synthesis and accumulation of cytoplasmic nucleocapsids (A particles), but extracellular virus was not released.

All MuLV+ cells showed complete cross-absorption. This is likely explained by the fact that MuLV infection usually results in the release of extracellular virus and the synthesis of all viral structural proteins; thus each cell contains the same complement of viral antigens.

Viral Antigens Produced in MTV-infected Cells

For determination of which antigens were being produced in MTV-infected cells, antisera prepared against isolated MTV antigens were utilized. These antisera (described in detail in Ref. 22) were prepared in rats against MTV proteins separated from ether-treated virus by Sephadex G-200 chromatography. They detect 5 serologically distinct antigens of MTV. These antigens have been designated *MTV-s1, s2, s3, s4,* and *s5. MTV-s1* and *s2* are the structural proteins of the viral nucleoid and are group-specific (*gs*) antigens for MTV. *MTV-s3* is the protein of the viral membrane and also is present in all MTV's. *MTV-s4* and *s5* are type-specific antigens of MTV and are presumed to reside on the spikes of the viral membranes.

Table 5 shows the results of immunofluorescence tests with these antisera on various MTV+ cells. From these tests, it is clear that the failure to find cross-absorption with all MTV-infected tissues was due to a limited synthesis of viral components in certain cell types. Thus, spleen cells, leukemia cells, and Leydig tumor cells produced the *s1* and *s2* antigens but not the *s3, s4,* and *s5* antigens. In several instances, the failure to find complete cross-absorption was attributable to type-specific variation in MTV's from different mouse strains. This does not, however, account for the limited synthesis of viral products observed within different tissues of the same mouse.

The Detection of MuLV and MTV Antigens in Normal Lymphoid Tissues of Mice

The direct examination of lymphoid tissues by immunofluorescence for viral antigens was found unsatisfactory because of the fluorescence of certain immunoglobulin-containing lymphoid cells (*i.e.*, fluorescence reactions were seen in cells with normal serum or 0.9% NaCl solution as controls for the viral-specific antisera). This difficulty was circumvented by absorption procedures. Immunofluorescence absorption was sensitive and reliable; in

Table 6

Quantitative IFA for MTV and MuLV antigens with tissue extracts of spleen, leukemias, and mammary tumors

Strains[a]	Tissue used for absorption	MTV+ leukemia (SL11)		MuLV+ leukemia (EδG2)	
		No. positive/ no. tested	Antigen titer	No. positive/ no. tested	Antigen titer
A	Spleen	12/14	1/1–1/8	0/3	
DBA/2	Spleen	2/7	1/1–1/2	8/12	1/1–1/2
DBA/2	Leukemia (ML+)	2/2	1/16	1/1	1/16
C3H/An	Spleen	3/4	1/1–1/2	1/5	1/1
C3Hf/Bi	Spleen			0/2	
GR	Spleen	0/7		0/7	
129	Spleen			0/2	
RF	Spleen			0/2	
BALB/c	Spleen	0/4		0/11	
C57BL/6	Spleen	0/3		0/12	
C57BL/6	Leukemia (EδG2)	0/1		1/1	1/32
C57BL/H-2^k	Spleen			0/4	
NZB	Spleen			2/2	1/4
C58	Spleen	0/1		14/14	1/2–1/8
AKR	Spleen	0/5		11/11	1/4–1/16
AKR/H-2^b	Spleen			5/5	1/4–1/8
C57BL/6 X AKR F_1	Spleen			6/6	1/1–1/2
BALB/c X AKR F_1	Spleen			4/4	1/1–1/4
AKR X BALB/C F_1	Spleen			3/6	1/1–1/2
C57B/6 X C58 F_1	Spleen			11/14	1/1–1/2
C58 X 129 F_1	Spleen			5/5	1/1–1/2
C57BL/6 X ASW F_1	Spleen			0/10	
BALB/c X C3H F_1	Spleen	0/2			
BALB/c X DBA/2 F_1	Spleen			0/6	
C57BL/6 X A F_1	Spleen	8/8	1/1–1/2		

[a] Age of mice ranges from 2 to 12 months.

fact, quantitative typing for viral *gs* antigen in the spleens of individual mice could be performed.[5] Table 6 summarizes these results.

MuLV. Typing by absorption showed MuLV antigens in the spleens of mice from high-leukemia-incidence strains (AKR and C58) and occasionally in the spleens of mice from some low-leukemia-incidence strains (NZB, C3H, DBA/2). The spleens of mice from other low-leukemia-incidence strains (C57BL/6, BALB/c, GR) were consistently negative. F_1 hybrid mice from parents of high- and low-leukemia-incidence strains (AKR or C58 X C57BL/6) showed levels of *gs* antigens intermediate between that of parental strains.

MTV. Since all MTV-infected cells did not show complete cross-absorption, a typing system was used that detected only the viral *gs* antigens (see Table 5). In this typing system, the spleens of mice from most high-mammary-tumor-incidence strains (A, DBA/2, C3H, and RIII) were positive; one exception was the GR mouse, which did not show viral antigens in any tissue other than the mammary gland or mammary tumor. The spleens of mice from low-mammary-tumor-incidence strains (C57BL/6, AKR, BALB/c) were invariably negative. F_1 hybrid mice from parents of high- and low-mammary-tumor-incidence strains (A and C57BL/6) showed levels of *gs* antigens intermediate between that of the parental strains. The amount of viral antigen in the spleen was greater in mice that were growing spontaneous mammary tumors.

Serological Relationship of MTV and MuLV to Other Oncornaviruses

Immunofluorescence and IFA tests showed that MTV was serologically unrelated to the avian, murine, rat, hamster, and feline leukemia-sarcoma viruses (Table 7).

MuLV was serologically unrelated to the avian leukosis-sarcoma viruses but showed at least 1 cross-reacting antigen with other mammalian leukemia-sarcoma viruses (Table 7).[6] This interspecies cross-reaction was seen only with

[5] Rat *anti-MuLV* serum at high dilution detects only the *MuLV-gs1* antigen. This was confirmed by fluorescence blocking tests, whereby the reactivity of rat *anti-MuLV* serum was completely abolished by pretreatment of test cells with monospecific guinea pig *anti-MuLV-gs1* serum (kindly provided by Dr. R. Gilden).

[6] Immunodiffusion studies (8, 21, 28) have demonstrated 2 types of *gs* antigens in the oncornaviruses: (*a*) the predominant *gs* antigen has viral species specificity; thus, all viruses of the same type (*i.e.*, leukemia-sarcoma viruses) from a given species showed serological cross-reaction; (*b*) another *gs* component was found common to all mammalian leukemia-sarcoma viruses (murine, feline, hamster, and rat). This antigen was described originally by Geering *et al.* (8) with MuLV antisera and was designated *MuLV-gs3*. More recently, Schafer *et al.* (28) have suggested the designation *interspec* (interspecies-specific) antigen.

Table 7

Demonstration of the serological cross-reaction between the mammalian leukemia-sarcoma viruses

Species	Cells tested	Viral antisera directed against				
		AvLV	MTV	MuLV	HaSV	FeLV
Chicken	AvLV-infected embryo cells	+[a]	–	–		–
	AvSV-infected embryo cells	+		–	–	–
Mouse	MTV-induced mammary tumor	–	+	–	–	–
	MuLV-induced leukemia	–	–	+	+	+
	MuSV-induced sarcoma	–	–	+	+	+
Hamster	HaSV-induced sarcoma	–	–	+	+	+
	HaLV-induced leukemia	–	–	+	+	+
	MuSV-induced sarcoma			–	–	–
	AvSV-induced sarcoma	+	–	–	–	–
Cat	FeLV-infected embryo cells	–	–	+	+	+
	FeSV-induced sarcoma			+	+	+
Rat	Novikoff hepatoma[b]		–	+		+
	Furth mammary tumor[b]		–	+		+
	AvSV-induced sarcoma	+		–		–
	MuLV-infected embryo cells	–	–	+	+	+

[a] +, cytoplasmic immunofluorescence.
[b] These rat tumors produce type-C virus particles.

low dilutions of antiserum (in general, lower than 1/64). Progressive dilution of MuLV antiserum resulted in a loss of the interspecies viral reaction and an emergence of only species-specific viral reactivity. Thus, at high dilution, MuLV antiserum reacted only with MuLV-infected cells; no cross-reaction with FeLV and HaSV was observed. This finding may be attributed to the presence of 2 antibodies of different specificity in disproportionate titers in the same antiserum. Antibodies to the interspecies antigen(s) were at a lower titer than the antibodies to the species-specific antigen(s); thus, the antiserum could be diluted past the end point of fluorescence for the interspecies antigen yet still retain reactivity for the species-specific antigen.

Antisera prepared against AvLV, HaSV, and FeLV also showed cytoplasmic fluorescence with cells infected with the homologous virus. These reactions were all species specific at dilutions near the end point of the antiserum, but at a low dilution the HaSV and the FeLV antisera also showed the interspecies reaction. Cross-absorption studies demonstrated that the interspecies antigen(s) from murine, hamster, and feline sources were serologically identical.

AvLV antisera did not react with cells infected with mammalian leukemia-sarcoma viruses, even at a low dilution.

DISCUSSION

Intracellular Localization of Viral Antigens. Antisera prepared against the *gs* antigens of AvLV, MuLV, MTV, HaSV, and FeLV give cytoplasmic fluorescence on virus-infected cells. Nuclear fluorescence has not been observed in any of these viral systems. Similar results have been reported for Rous sarcoma virus (7, 14, 25, 30), MuLV (3, 15, 17, 31), and murine MTV (4, 5, 11, 22). In contrast, nuclear fluorescence also has been described with Rous sarcoma virus (16, 18, 23, 26) and MuLV (6, 15, 27). This discrepancy is possibly due to the fact that the antisera used by different investigators detect different viral antigens; however, a second factor causing the discrepancy observed between the results of different investigators on the intracellular localization of viral structural antigens may be technical. Heterologous antisera were used in most investigations where nuclear fluorescence was reported, and in all cases these antisera were absorbed *in vitro* prior to dilution. In our studies, we have found it impossible to remove all nonviral staining by *in vitro* absorption of undiluted serum, even if the absorption procedure was repeated several times. Since in many instances we have observed nonviral nuclear fluorescence with inadequately absorbed heterologous antisera, it seems possible that some of the nuclear fluorescence reported by others may have nonviral origins.

Typing of Individual Mice by the IFA Test. The typing of mouse strains for *MuLV-gs* antigens by IFA correlates with similar studies where either the cytotoxic test was used to detect GCSA (24) or the immunodiffusion (1, 9, 20) and complement fixation (13) tests were used to detect *MuLV-gs* antigens. Three categories of mice are identified on the basis of the antigen expression in the spleen.

1. Mice with high levels of *MuLV-gs* in the spleen. This correlates with the overt production of MuLV in these mice. Examples are the high leukemia strains AKR and C58. The usual titer of *MuLV-gs* antigen in IFA tests ranges from 1/4 to 1/8.

2. Mice with intermediate levels of *MuLV-gs* in the spleen. This is found in some low-leukemia-incidence strains (*i.e.*, DBA/2 and C3H) that show viral activation with aging. The titer of *MuLV-gs* antigen ranges from 1/1 to 1/2 in positive mice. The same intermediate levels are also found in F_1 hybrids between mice of high-leukemia strains (AKR, C58) and mice of low-leukemia strains (C57BL/6).

3. Mice with no *MuLV-gs* antigens detectable in the spleen.

Mice from certain low-leukemia strains, such as BALB/c and C57BL/6, do not show detectable *MuLV-gs* antigen in their spleens.

As to whether the concentration of *gs* antigens in the lymphoid tissues is a reflection of the number of antigen-producing cells or the amount of antigen produced by each cell, it appears that both factors play a role. Thus, in direct fluorescence tests, the percentage of *gs* positive cells is higher in spleens of AKR mice than in spleens of C57BL/6 X AKR F_1 mice. Furthermore, the intensity of fluorescence per cell appears greater in the spleens of AKR mice than in the spleens of F_1 hybrid mice.

In contrast to MuLV, *MTV-gs* antigens are observed only in the lymphoid tissues of mice from high-mammary-tumor strains (with the exception of the GR strain, see below). Mice of low-incidence strains are invariably negative for *MTV-gs* antigen in their spleen. Three categories of mice are identified on the basis of antigen expression in the spleen.

1. Mice with high levels of *MTV-gs* and no *MuLV-gs* in the spleens. The one example is the A strain. This correlates with the presence of *MTV-gs* and the absence of *MuLV-gs* in mammary tumors and milk of this strain.

2. Mice with intermediate levels of *MTV-gs* and intermediate or no *MuLV-gs* in the spleens. This is found in C3H, DBA/2, and C57BL/6 X A F_1 mice. The level of the F_1 hybrid is intermediate between the high parent (A) and the low parent (C57BL/6).

3. Mice with no *MTV-gs* detectable in the spleens. Mice from all low-mammary-cancer strains and one high-mammary-cancer strain (GR) do not show detectable *MTV-gs* in their spleens.

Daams *et al.* (4, 5) also have used the immunofluorescence technique and report MTV antigens in the spleens of mice from high-mammary-tumor strains (C3H, GR, BALB/cfC3H) but not in spleens from low-mammary-cancer strains (C57BL and 020). They observed fluorescence on both the cell surface and in the cytoplasm of virus-infected cells; in addition, these antigens were found by absorption techniques in density-gradient-purified MTV. As mentioned above, there are difficulties in interpreting indirect immunofluorescence reactions with spleen cells, since controls of normal serum or 0.9% NaCl solution also give positive reactions. Thus, in a previous study we have expressed reluctance to attribute immunofluorescence in spleens of MTV+ mice as viral specific (22). With IFA, we can exclude nonspecific fluorescence and have shown that MTV antigens occur in the spleens of mice from certain MTV+ strains. However, our results differ from those of Daams with regard to the strain and tissue distribution of these antigens (*i.e.*, all tissues except mammary gland and tumor tissues of GR mice were consistently MTV negative in our hands, although they were found to be positive by Daams). Since the results reported here show that viral membrane components are not synthesized in MTV-infected spleen cells, the most likely explanation of the cell surface antigen reported by Daams *et al.* (4, 5) is that this antigen is not a virion structural protein but is rather a virus-induced cell surface antigen. This would be similar to the ML antigen described by Stuck *et al.* (29). The presence of a cell surface antigen in the "purified" virus preparations used for immunization and absorption studies could be due to contaminating vesicles, these being commonly found in density-purified viral preparations. This possibility can be entertained since we know that density-gradient-purified MuLV contains GCSA as detected by absorption techniques (E. Stockert, personal communication), although the GCSA antigen is a nonstructural, virus-induced antigen (2).

ACKNOWLEDGMENTS

We extend our sincere thanks to Dr. L. J. Old for his helpful and exciting discussions of the work and to Dr. Y. Hirshaut for introducing the immunofluorescence method to us. We also thank Dr. E. Fleissner for the contributions of viral antigens and Dr. Marina Beya for making the spleen preparations.

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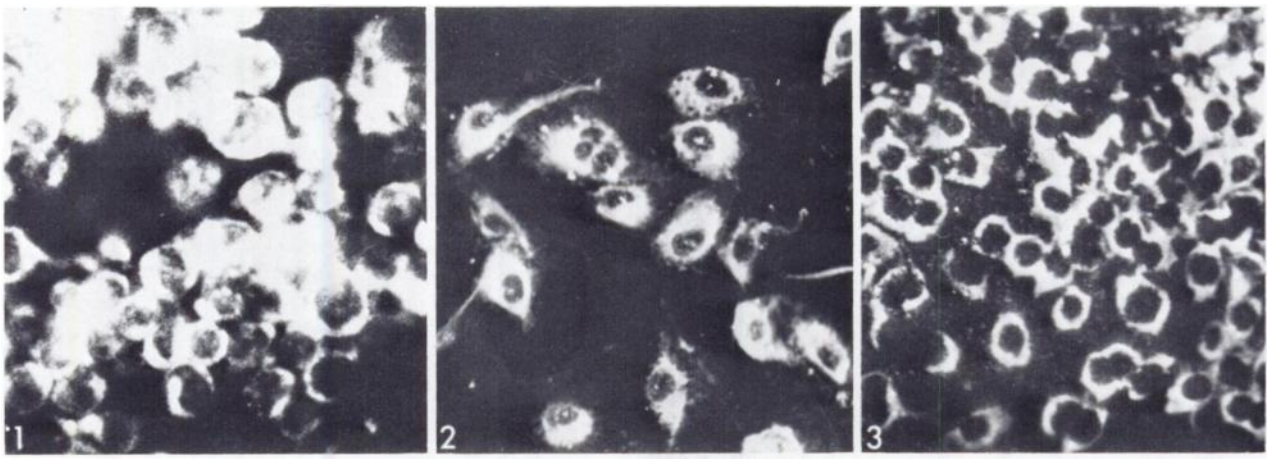

Figs. 1 to 3. In all cases, the cytoplasm was stained green and the nucleus red (because of the applied counterstain, Evan's blue). In negative reactions (not shown), both the cytoplasm and the nucleus were stained red.

Fig. 1. Immunofluorescence reaction showing the cellular deposition of *MTV-gs* antigens in a ML+ leukemia of the DBA/2 strain.

Fig. 2. Immunofluorescence reaction showing the cellular deposition of *MuLV-gs* antigens in a culture of rat cells infected with MuLV (Gross). The antiserum used was rat *anti-MuLV*.

Fig. 3. Immunofluorescence reaction showing the cellular deposition of the *gs3* (interspecies) antigen in MuLV-infected rat cells. The antiserum used in this test was prepared against FeLV.