Atypical Cells in the Peripheral Blood of Chickens Exposed to Marek's Disease Agent

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SUMMARY

Atypical cells, containing opaque blue cytoplasmic globules when stained with Wright's or May-Grünwald-Giemsa stain, were observed in Gamefowl (GF-WSU) experimentally exposed by contact to a classical Marek's disease (MD) agent, and in White Leghorn chickens (RPL Line 7) in which group "spontaneous" cases of classical MD developed. Such cells were absent in uninfected control birds.

The cells were most readily demonstrated in stained films of white blood cells of the peripheral blood after 24-hour culture in vitro.

The atypical cells were present in the peripheral blood of chickens exposed to MD, but in numbers so few that the cells were rarely detected in direct blood smears.

INTRODUCTION

Intracytoplasmic globule formations in lymphocytes have been reported in certain neoplastic diseases of man. Rappaport and Johnson (13) described cytoplasmic inclusions resembling Russel bodies in lymphocytes of lymph nodes from malignant lymphomas. Recently Laszlo *et al.* (6) reported observations on cytoplasmic globules in lymphocytes of peripheral blood, bone marrow, and lymph nodes of a patient with lymphocytic leukemia.

This paper reports preliminary observations of atypical cells containing cytoplasmic globules in the peripheral blood of chickens exposed to classical Marek's disease (MD) agent.

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MATERIALS AND METHODS

MEDIA

Medium 199 was prepared by adding 10.0 ml of TC Medium 199 (10X)[1] to 90.0 ml of triple distilled water plus 1.0 ml of stock antibiotic solution which contained 10,000 units of penicillin, 10,000 μg of streptomycin, and 250 μg of fungizone[2]. No bicarbonate was added for white blood cell cultures.

Growth medium for white blood cell cultures was prepared by mixing an equal volume of Medium 199 and autologous plasma. The pH of the medium was usually 7.3-7.4 without adjustment.

HEPARIN[3]

Each ml contained 1,000 U.S.P. units of heparin sodium, 9.0 mg benzyl alcohol, and 9.0 mg NaCl. Two tenth milliliter was used for 10.0 ml of blood.

BACTO-PHYTOMAGGLUTININ M[4]

The content of each vial was dissolved in 5.0 ml of sterile triple distilled water and stored at 4°C. To each 10.0 ml of heparinized blood, 0.2 ml of this solution was added.

CHICKENS

Gamefowl (*Gallus domesticus*) and RPL Line 7 White Leghorn chickens were used in this study. Gamefowl (GF-WSU) used in this study were the progeny of the Game-

[1]Difco Laboratories, Detroit, Michigan.
[2]Amphotericin B, E. R. Squibb & Sons, New York.
[3]Heparin sodium sterile solution, The Upjohn Co., Michigan.
[4]Difco Laboratories, Detroit, Michigan.

fowl breeder flock maintained by this department (4). These breeders were free of RIF (resistance inducing factor) and RVNA (Rous sarcoma virus neutralizing antibodies) when tested by RIF (14) and RVNA (15) tests.

RPL Line 7 White Leghorns were hatched from eggs made available to us through courtesy of the Regional Poultry Research Laboratory, East Lansing, Michigan.

The progeny from Gamefowl breeders and RPL Line 7 White Leghorns were hatched in the following manner: on the 18th day of incubation the eggs were washed in a detergent solution, rinsed twice in water, dipped in a 1:5,000 Roccal[5] bath, and hatched in separate isolated incubators that had been fumigated three times with formalin-potassium permanganate.

Control and exposed groups of chickens were attended by different caretakers, who wore separate clean outer clothing, caps, and boots for each group of birds. O-syl[6] disinfectant was used for cleaning footgear.

RIF and RVNA Tests

RIF and RVNA tests were essentially based on the method of Rubin (14,15) as modified by Dr. F. Piraino of the Regional Poultry Research Laboratory, East Lansing, Michigan (unpublished data).

Embryonated Gamefowl eggs from the RIF-free Gamefowl breeders were used for all the chick embryo fibroblast (CEF) cultures. Most of the CEF cultures from this breeder flock proved to be C/O phenotypes (3).

For RIF and RVNA tests, both subgroups A and B pseudotypes of Bryan high-titer strain of Rous sarcoma virus (BH-RSV) were used, namely, BH-RSV (RAV-1)[7] and BH-RSV(RAV-2)[7] which contained 2.7 x 10^7 ffu (focus forming unit) and 3.2 x 10^5 ffu per ml of virus material, respectively.

Diagnosis of MD

The diagnosis of MD was determined on the basis of microscopic lesions in nerves (brachial plexuses, coeliac plexus, and sciatic plexuses) and gonads as described by Payne and Biggs (11).

White Blood Cell Cultures

The 24-hour white blood cell (WBC) cultures were prepared by Nowell's method (9) with some modifications. From each donor bird, 10.0 ml of blood were drawn by venipuncture into a 20.0 ml syringe containing 0.2 ml (200 U.S.P. units) of heparin sodium. After thorough mixing, this blood was gently discharged into a screw-capped tube (16.0 x 125.0 mm) containing 0.2 ml of phytohemagglutinin M and mixed well by gentle shaking. The tube was allowed to stand in an incubator (37°C) for 30 minutes and then centrifuged at 500 r.p.m. (40 x G) for 90 seconds (International Centrifuge, Size 2, Model K) to obtain plasma without a buffy layer. This plasma layer contained WBC (plasma-WBC) which consisted of 98% lymphocytes and 2% of other WBC together with a few erythrocytes. The plasma layer was then aspirated and kept at 4°C until the cells were ready for culture. The volume of plasma-WBC collected was usually 0.2 ml or more. If it were less than 0.2 ml, the tube of blood was gently reshaken and recentrifuged. The plasma-WBC volume was adjusted to 1.1 ml by adding autologous plasma and 0.1 ml was used for each cell count. The WBC suspension was then diluted with autologous plasma to give a cell concentration of 1 x 10^7 per ml. One milliliter of this suspension was then mixed with an equal volume of Medium 199 and seeded in a Leighton tube. The pH of the culture was 7.3-7.4 without adjustment. Following incubation in a slant position for 24 hours at 37°C, the cells in suspension were transferred, without disturbing the cells (mostly granulocytes, monocytes, and clumped thrombocytes) attached to the glass, to a screw-capped tube and centrifuged at 1000 r.p.m. (80 x G) for 5 minutes.

The cell deposits consisting of 98% or more lymphocytes were spread on a slide by the direct blood smear method and stained with Wright's or May-Grünwald-Giemsa (MGG) stain, and then completely scanned for atypical cells. Through the use of a fixed size of wire loop (1.0 mm in outer diameter, Brown & Sharpe Gauge No. 24), four loopfuls of cell deposits were used

[5]Benzalkonium chloride, Winthrop-Stearns, Inc., New York.

[6]Lehn and Fink Products Corporation, Toledo, Ohio.

[7]Kindly provided by Dr. P. S. Sarma who originally obtained the seed viruses from Dr. P. K. Vogt.

to deliver approximately 5.0 x 10^5 cells for each smear. Extreme care was exercised to minimize the variation in numbers of cells delivered. For histochemical staining, periodic acid-Schiff (PAS) (7) and Sudan black stains (8) were employed.

EXPERIMENTS

Trial 1. — Twenty-five Gamefowl chicks were exposed experimentally by contact at 5 days of age to Gamefowl clinically affected with Gamefowl (GF) strain of classical MD (4). Thirty-two siblings kept under isolation served as controls.

WBC cultures from samples of both the control and exposed birds were examined for atypical cells at 6 weeks and 12 weeks post-exposure. Eleven exposed birds were sampled twice.

Trial 2. — Of 29 twelve-week old Gamefowl siblings negative for atypical cells, 16 were exposed by contact to clinical cases of Gamefowl MD, and 13 birds were kept under isolation as controls.

WBC cultures made from both the control and exposed birds 2, 6, and 12 weeks later were examined for atypical cells. Both the control and exposed birds were sacrificed immediately after the last WBC culture at 12 weeks post-exposure for histopathological examinations for MD infection.

Trial 3. — WBC cultures from 13 twenty-two-week old chickens in a pen of 72 White Leghorns (RPL Line 7) were examined for atypical cells. Several "spontaneous" cases of MD developed in these birds.

Trial 4. — In order to determine if MD-exposed birds with the atypical cell were infected and "viremic", heparinized blood samples were taken from four of the MD-exposed birds of Trial 2 and inoculated into separate groups of Gamefowl chicks.

Each blood specimen was inoculated intra-abdominally (0.3 ml) into each of a group of 5 day-old Gamefowl chicks which were maintained by group in separate modified Horsfall (HF) cages. Two groups of uninoculated siblings served as controls, one group of 5 chicks was kept in a modified HF cage in the HF room, while another group of 10 chicks was kept in a modified HF cage in a separate isolation building. The former group served to check for cross transmission of MD between HF cages, and the latter to check possible egg-borne transmission to chicks used for inoculation. These inoculated and control chicks were observed for clinical cases of MD and sacrificed at six weeks post-exposure for histopathological examinations.

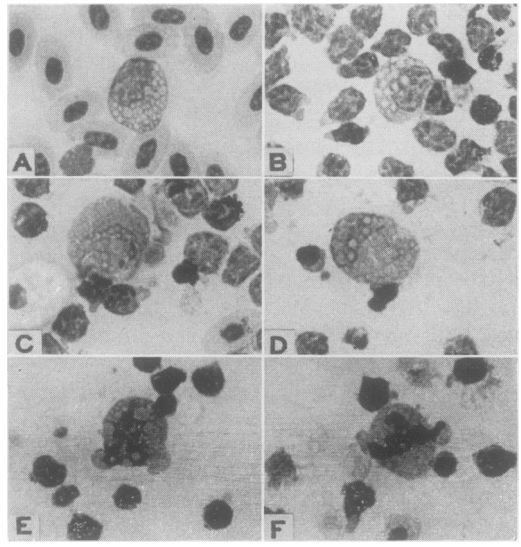

Fig. 1. "Blue globule cells" (BG cells) in chickens (Gamefowl) exposed to classical Marek's disease agent. A BG cell in direct blood smear (A) and in the 24-hour white blood cell culture (B, C, and D). Note the cytomplasms packed with opaque blue staining globules (C and D). BG cells in mitosis (E and F). Wright's stain. X 911.

RESULTS

Atypical cells as described below were present in the peripheral blood of chickens exposed to MD (Fig. 1A), but in numbers so few that the cells were rarely detected in direct blood smears. When white blood cells from MD exposed birds were cultured for 24 hours *in vitro*, the efficiency of detecting these cells increased, probably due to mitotic activity of some of the atypical cells (Fig. 1, E and F). Therefore, in this study, we examined WBC of the peripheral blood for the presence of atypical cells after 24-hour culture.

The atypical cells observed in stained smears contained cytoplasmic globules, which exhibited an opaque blue staining with either Wright's or MGG stain (Fig. 1). These globules, however, did not stain with either PAS or Sudan black stain. The atypical cell itself had no PAS-positivity and was not reactive for lipid.

These atypical cells were designated as "Blue globule cells" (BG cells). The BG

cells varied somewhat in size and morphology. Some of them were morphologically indistinguishable from lymphocytes (Fig. 1, A and B), while others were 1.5 times as large as a normal granulocyte and had a relatively large irregularly shaped nucleus without a visible nucleolus.

The nucleus, often located eccentrically, was less pachychromatic and exhibited a variable degree of chromatin clumping. The abundant basophilic non-granular cytoplasm was usually obscured by many opaque blue globules (Fig. 1, C and D).

Observations on BG cells from chickens experimentally and naturally exposed to a classical MD agent (Trials 1, 2 and 3) are summarized in Table I.

TRIAL 1

At 6 weeks post-exposure, 12 (66%) of 18 birds, including 5 clinical cases, had from 1 to 21 BG cells in smears of their respective WBC cultures. At 12 weeks post-exposure, 8 (44%) of 18 birds, including 4 clinical cases, and 11 birds which had been tested at 6 weeks post-exposure, were positive for BG cells (3 to 59 cells per smear). None of the control birds were positive for BG cells. There was no noticeable difference in number of BG cell-positive birds in the clinically affected and the clinically normal exposed groups.

Ten of 18 exposed birds randomly selected at 6 weeks post-exposure, were RIF negative when their plasmas were tested by RIF test using both BH-RSV(RAV-1) and BH-RSV(RAV-2). Of 18 exposed birds, 5 birds died of MD between 6 and 12 weeks post-exposure with gross and microscopic changes in the peripheral nerves (two of which were positive for BG cells at 6 weeks post-exposure). Visceral MD tumors are relatively uncommon in chickens exposed to the GF strain of classical MD.

TRIAL 2

At 2 weeks post-exposure, 13 (81%) of 16 contact exposed birds were positive for BG cells, while all control birds remained negative.

The prevalence of BG cells in the exposed birds in Trial 2 (Table I) at 6 weeks and 12 weeks post-exposure was essentially the same as that observed in Table I; 10 (62%) of 16 birds at 6 weeks, and 8 (53%) of 15 birds at 12 weeks post-exposure were positive for these cells. It appeared that more birds were positive in the early stage of infection.

Despite the absence of clinical MD cases and an absence of gross lesions in these 15 birds when they were killed following the last WBC cultures, 13 birds (86%) exhibited microscopic lesions characteristic of MD (11), indicating the presence of subclinical infection. The lesions were characterized by minimal to moderate degrees of infiltration of small and medium lymphoid cells together with limited numbers of plasma cells and Marek's disease cells (11). All of these birds were positive for BG cells at some time during the experimental period.

TABLE I. Occurrence of Atypical Cells with Opaque Blue Staining Cytoplasmic Globules (BG Cell) in the 24-hour Cultures of White Blood Cells of Chickens Exposed to Classical Marek's Disease Agent.

	Group and treatment	Strain of chicken	No. of birds	Numbers of BG cell-positive birds after exposure			
				Pre-exp.	2 wks.	6 wks.	12 wks.
Trial 1§							
	Contact exposed	GF§§	25	—	—	12/18* (66%)	8/18 (44%)
	Control	GF	32	—	—	0/14 (0%)	0/18 (0%)
Trial 2							
	Contact exposed	GF	16	0/16 (0%)	13/16 (81%)	10/16 (62%)	8/15 (53%)**
	Control	GR	13	0/13 (0%)	0/13 (0%)	2***/13(15%)	1***/12(8%)**
Trial 3							
	Natural exposure	WL#	13				8/13 (61%)##

§Only a portion of the birds were sampled for WBC culture each time.
§§Gamefowl.
*Numbers of positive birds/numbers of birds tested.
**One bird died due to cannibalism.
***Birds considered infected with MD when examined histologically.
#RPL Line 7 White Leghorns.
##Time of exposure unknown.

In Trial 2, during the 12-week experimental period, 3 control birds became positive for BG cells (Table I), although none of them showed any clinical signs suggestive of MD. Upon histopathological examination MD lesions closely resembling mild A-type lesions (11) but without noticeable plasma cells, were noted in two birds, and suspicious lesions in one.

TRIAL 3

At the time of this trial, one "spontaneous" case of MD developed in this flock of 72 White Leghorn chickens when 22 weeks old, and one more case developed in the next 3 months. Each clinical case of MD was confirmed by histopathological examination, as well as by inoculation of heparinized blood from the affected birds into Gamefowl chicks. The clinical and pathological expressions of the disease, as judged by low morbidity, mortality, and absence of lymphoid tumors in visceral organs, conformed to classical MD (2,10).

In this trial, 8 (61%) of 13 birds, including one clinical case, had from 1 to 49 BG cells per smear in the respective WBC cultures. These birds were all RIF negative when their plasmas, collected at the time of WBC culture, were tested by RIF test, using both subgroups A and B pseudotypes of BH-RSV.

TRIAL 4

These MD-exposed birds of Trial 2 were found to be infected and "viremic" at two weeks post-exposure, when 13 of 16 birds were positive for BG cells.

The incidence of clinical and histopathological cases of MD in each of a group of five day-old Gamefowl chicks, which had been inoculated with each blood specimen taken from four MD-exposed birds of Trial 2 at two weeks post-exposure, are summarized in Table II.

All four blood donor birds were "viremic", three of them were positive for the BG cell.

In addition, limited numbers of Gamefowl exposed experimentally by contact to CR strain[8] of acute MD agent, were also examined for the BG cell. No BG cells were observed in the 24 WBC cultures prepared at 6 weeks and 12 weeks post-exposure from 20 contact exposed Gamefowl which included 6 clinical cases.

[8]Kindly provided by Dr. B. R. Burmester.

DISCUSSION

Although the significance of the BG cells is not known, it appears that they are associated with classical Marek's disease.

TABLE II. Incidence of Marek's Disease (MD) in Gamefowl Chicks Inoculated with Blood of Gamefowl Exposed for 2 Weeks to Classical MD Agent.

Blood donor	BG cell status	Cases of MD in inoculated Gamefowl chicks* Clinical cases	Histopathological cases
1	−	0/5** (0%)	5/5 (100%)
2	+	1/5 (20%)	4/5 (80%)
3	+	0/5 (0%)	5/5 (100%)
4	+	2/5 (40%)	5/5 (100%)
Control#		0/5 (0%)	0/5 (0%)
Control##		0/10 (0%)	0/10 (0%)

*Observations 6 weeks after inoculation.
**Numbers of MD positive birds/numbers of birds inoculated.
#Control (uninoculated chicks) for cross transmission between Horsfall cages.
##Control (uninoculated chicks) for possible egg-borne transmission of MD.

The protein nature of these globules was assumed, since they stained blue with MGG stain, but did not stain with Sudan black or with periodic acid-Schiff stain.

The possible effect of phytohemagglutinin on the formation of the BG cells was considered. However, these cells were observed in direct blood smears as well as in WBC cultures which were not treated with phytohemagglutinin.

The reason that the BG cells could be detected with greater efficiency in WBC smears following 24-hour culture may be due to mitotic stimulation (Fig. 1, E and F) by phytohemagglutinin. Nowell (9) showed that phytohemagglutinin had the ability to initiate mitosis in cultures of leucocytes isolated from peripheral blood of man.

Failure to detect the BG cells in those birds exposed to CR strain of acute MD agent may be due to differences in the biological characteristics and pathogenesis of the various strains (isolates) of MD agents (1,2,5,10,12,16-18).

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