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犬免疫溶血性貧血之致病機轉與靜脈注射人類免疫球蛋白治療評估之研究(1/2)

Abstract

Immune-mediated hemolytic anemia (IMHA) is a common cause of severe anemia in dogs. IMHA can be diagnosed by detecting spherocytosis or autoagglutination in blood smear, or by positive Coombs' test. The aim of study was to establish the Coombs' assay system and to evaluate the therapeutic efficacy of intravenous administration of human immunoglobulin (hIVIG) in dogs with IMHA. The Coombs' test was performed on anticoagulated blood and used species-specific antiserum prepared against immunoglobulins (IgG and IgM) and complement (anti-C3). Six dogs with IMHA (were confirmed by positive Coombs' test or autoagglutination) were collected and treated with hIVIG intravenously (1g/kg) in 10- to 24-hour period. Complete blood counts were repeated on days 1 through 7 and 14, 30, 60, 90, 120, 150, and 180 days after treatment. Four of six dogs are survival over 6 months and still alive now. One of them was survival 4 months, and another only 3 weeks after administration of hIVIG. Before therapy, the mean of hemoglobin concentration (Hb) was 5.43 g/dl, hematocrit (Hct) was 17.70 %, and red cell count (RBC) was 2.16× 10^6/î L. The levels of Hb, Hct and RBC were elevated to 9.03 g/dl, 31.45 %, 3.57 10^6/î L 7 days after therapy. Hematological data of four surviving dogs recovered to normal range 30 days after therapy. During the therapy, all of the dogs showed no signs of immediate and delayed side effect, and the clinical signs were improved one week after therapy. In conclusion, we have established Coombs' assay system to screen clinical suspected cases of IMHA; in addition, the results intravenous administration of human immunoglobulin suggested, it is a good alternative agent in treatment of IMHA.

INTRODUCTION

Immune-mediated hemolytic anemia (IMHA) is one of the common dog diseases causing severe anemia. Etiology is classified as primary and secondary IMHA. Diagnosis can be confirmed by detecting autoagglutination or spherocytosis in blood smear, or by positive Coombs' test. Immunosuppressive agents are most commonly used in treatment of canine IMHA. But sometimes this disease is difficult to control with immunosuppressive drugs, and those side effects may be very severe. Recently intravenous administration of human immunoglobulin (IVIG) in canine IMHA was also studied. Preliminary study showed a potential effect and without any obvious side effect.

The immunopathogenic mechanisms underlying idiopathic IMHA are not yet clearly studied, according to the following studies, regulatory of cytokines may play

an important role. Interleukin (IL)-2-deficient mice develop a severe systemic autoimmune disease including IMHA that is prevented by IL-2 treatment *in vivo*, suggesting that IL-2 is required for the development of self-tolerance. In anti-erythrocyte autoantibody transgenic mice, activation of peritoneal B-1 cells through administration of lipopolysaccharide, IL-4, IL-5 and IL-10 triggers IMHA. New Zealand Black (NZB) mice, which spontaneously develop an autoimmune disease resembling human IMHA and systemic lupus erythematosus (SLE), showed several immunological abnormalities, and the development of RBC autoantibody is associated with an unexpected Th-1 cytokine-dominated response. In the study to investigate patterns of *in vitro* cytokine production by unstimulated and mitogen-stimulated whole blood cultures from human IMHA patients, they found that a predominant Th-2-like profile might play a role in the immunopathogensis of IMHA.

The therapeutic mechanisms of IVIG are also not clearly proved. There are many hypotheses, including 1) inhibition of phagocytosis by blocking Fc receptor, 2) B-cell activation, 3) Change in T-cell distribution and function in part related to anti T-cell receptor antibodies, 4) Down-regulation of interleukin production and modulation of Fc-receptor expression, 5) membrane stabilizing and immune complex solubilizing effect and, 6) Inhibition of deposition of early complement activation products onto target surfaces.

This study was designed to investigate expression of cytokine mRNA from peripheral blood mononuclear cells (PBMC) before and after therapy with hIVIG in IMHA dogs.

MATERIALS AND METHODS

Diagnosis and Therapy of Canine IMHA

1. Diagnosis base on clinical signs, autoagglutination, spherocytosis and positive Coombs' test.
    1. Direct: the blood samples were collected in EDTA and centrifuged to separate blood cells and plasma for 10 min in 3400 rpm. The RBCs were washed with 0.9% saline 4 times and added to 96 wells plate. It was performed at 37 -canine immunoglobulin G and 4 (IgG), complement component C3, goat anti-canine immunoglobulin M (IgM), and rabbit anti-canine IgG.
    2. Indirect: the separated plasma of test dogs were added to the same volume of washed RBCs from normal dog and the mixture was incubated in 37 for 30 r
    followed up the procedure of direct
2. Intravenous administration with human immunoglobulin (1 g/kg) was given for

IMHA dogs.

Preparation of Canine PBMC

1. Blood samples from IMHA dogs were collected in pre and 1, 2, 4, 8 weeks post hIVIG therapy.
2. Blood buffy coats were centrifuged and suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO-BRL).
3. PBMC was collected through Ficoll-Paque® (Pharmacia) isolation and suspended in RPMI 1640 medium containing 10% FBS.
4. Two-hour cultures were stimulated with 2ì g/mL concanavalin A (ConA)

RNA Isolation and cDNA Synthesis

1. Total RNA (intact and mitogen-stimulated) was extracted from PBMC by TRIzol (GIBCO-BRL) purification.
2. Total cellular RNA (4ì g) was reverse transcribed by using oligo (dT) primer (heat mixture to 70 (GIBCO-BRL) at 42 for 50 min, and terminated mixture at 70 for 15 min.

Polymerase Chain Reaction

1. The 1:10 dilution cDNA (1ì L) as template for amplification.
2. Hot-star method. *Taq* DNA polymerase (Promega) was added after incubation of the mixture at 95 for 5 min.
3. Single round PCR consisted of 28 cycles (30 s template denaturation at 94 30 s primer binding at 55), with an initial 5 min denaturation at 94 and an extra 5 min elongation at 72 for completion.

Semiquantitative Assay for Cytokine Gene Expression

1. Electrophoresis through a 1.5% agarose gel (Amresco®, Solon, Ohio).
2. Recorded by a gel video system and analyzed by image analysis software (AlphaImager™ documentation and analysis system).
3. Cytokine gene expression was measured and standardized by the amounts of internal house-keeping gene G3PDH expression in each sample.

Table 1. Sequence of primers for amplification of canine cytokine-gene transcripts

	Primer Set	Product
IL-2	5’ primer: 5’ GTG CGC CTA TTA CTT CAA GCT C 3’	345 bp
	3’ primer: 5’ GCT GTC TCG TCA TCA TAT TC 3’	
IL-4	5’ primer: 5’ GCA CTC ACC AGC ACC TTT GTC 3’	318 bp
	3’ primer: 5’ GCC TTT CCA AGA AGT CTT TCA G 3’	
IL-10	5’ primer: 5’ CCT GGG TTG CCA AGC CCT GTC 3’	484 bp
	3’ primer: 5’ GGG AGT TGA GGT ATC AGA G 3’	

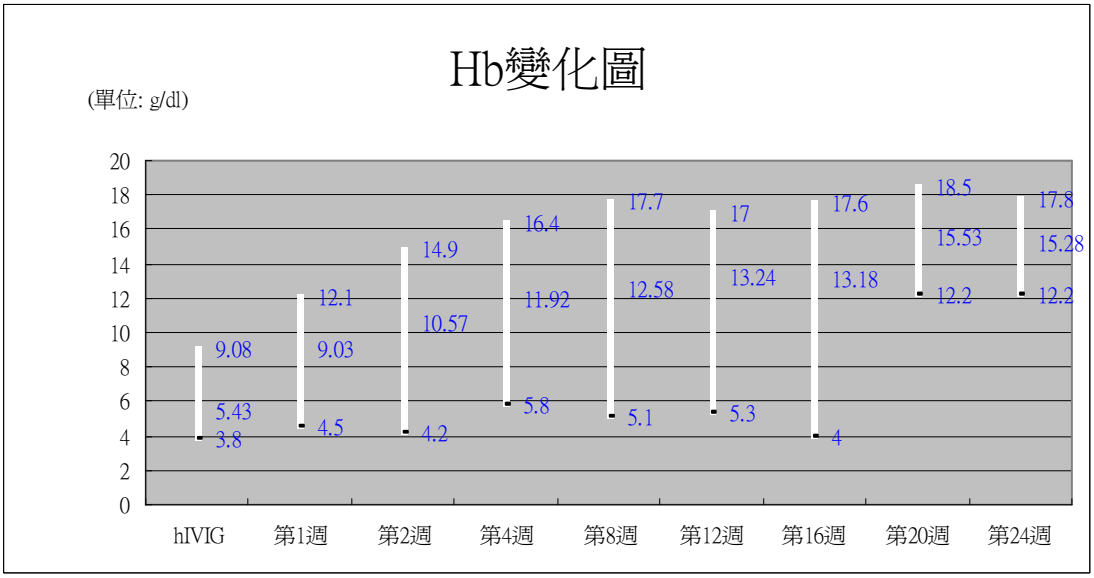
IFN-ã	5’ primer: 5’ GCA AGT AAT CCA GAT GTA TCG G 3’	415 bp
	3’ primer: 5’ CAA ATA GTG CTG GCA GGA TGA CC 3’	
G3PDH	5’ primer: 5’ CCT TCA TTG ACC TCA ACT ACA T 3’	400 bp
	3’ primer: 5’ CCA AAG TTG TCA TGG ATG ACC 3’	

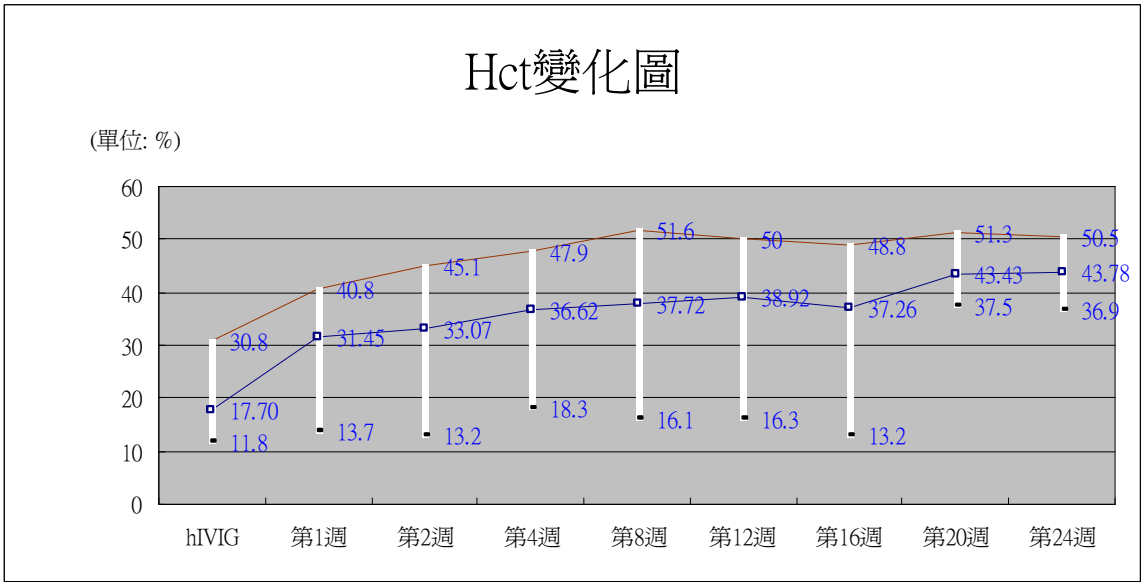
PRELIMINARY RESULTS

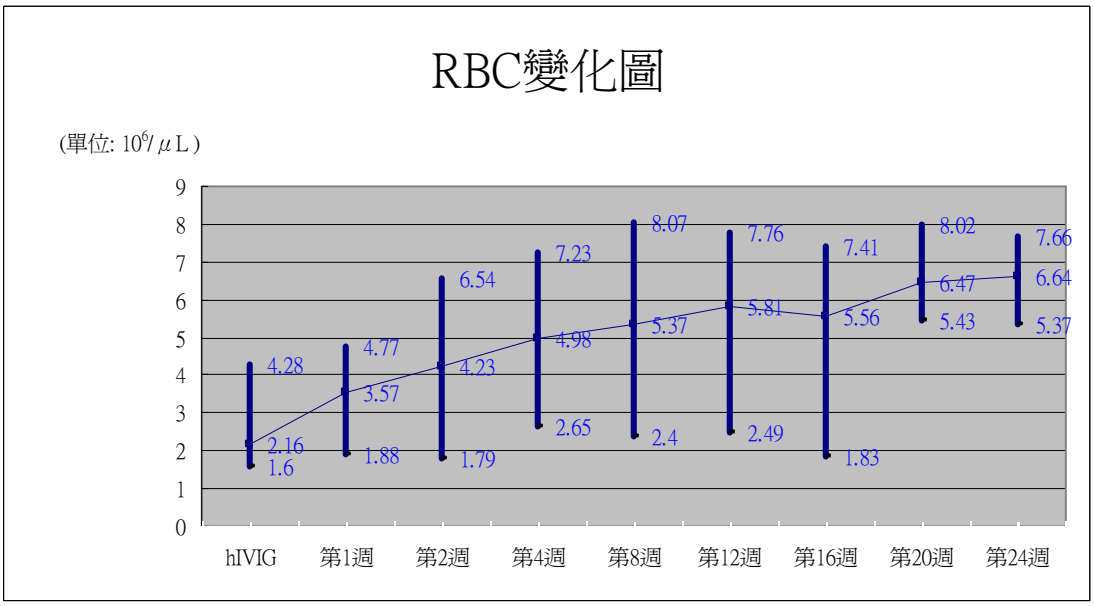
Until now six dogs with IMHA were collected in this study. Diagnosis had been confirmed by detecting spherocytosis or autoagglutination in blood smear and by positive Coombs’ test. Four of six dogs are survival over 6 months and still alive now. One of them was survival 4 months, and another only 3 weeks after administration of hIVIG. Before therapy, the mean of hemoglobin concentration (Hb) was 5.43 g/dl, hematocrit (Hct) was 17.70 %, and red cell count (RBC) was 2.16× 10^6/î L. The levels of Hb, Hct and RBC were elevated to 9.03 g/dl, 31.45 %, 3.57× 10^6/î L 7 days after therapy. Hematological data of four surviving dogs recovered to normal range 30 days after therapy. During the therapy, all of the dogs showed no signs of immediate and delayed side effect, and the clinical signs were improved one week after therapy.

All dogs administered with human intravenous immune globulin (IVIG, 1 g/kg) during 10- to 48-hour period. Prednisolone was not given or not continued to give after IVIG administration. CBC and RPI were repeated on days 1 through 7, 14, 30, 60, 90, 150 and 180 days after treatment.

In the six dogs Hb, RBC counts and Hct increased with the time. Four of them didn’t need any additional treatment more and showed very good prognosis. Two of them were died with other complication.







The data of expression of cytokines are in progress.

Conclusion

- We have established direct and indirect Coombs' tests in clinical usage.
- Evaluation of the therapeutic efficacy of IVIG, we observed that,
  1. During the therapy, all of the dogs showed no signs of immediate and delayed side effect.
  2. The clinical signs were improved one week after IVIG therapy.
  3. Hematological data of four surviving dogs recovered to normal range 30 days after therapy.
- According the results and observation, intravenous administration of human immunoglobulin can be a good alternative agent in treatment of IMHA. But we

still need more cases to support the conculsion.